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THE EFFECT OF PHENOLIC MEDIATORS IN OXIDOREDUCTASE CATALYZED CONVERSION OF LIGNIN MODEL COMPOUNDS AND SYNTHETIC LIGNIN

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ACADEMIC DISSERTATION

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ABSTRACT

Eco-efficient biotechnological applications are important innovations for modern industrial developments, such as production of chemicals and fuels from renewable biomass. Lignocellulosic biomass is the only sustainable organic resource that can be converted to both energy and various value-added products. The effective use of wood and plants for feedstock in biorefineries is in most cases dependent on the plant cell wall recalcitrance and especially the extent of lignification of the cell wall. Oxidative enzymes such as laccases and manganese peroxidases (MnPs), provide interesting possibilities for plant biomass modification as they are the key catalysts in modification and degradation of the most recalcitrant component of the lignocellulose biopolymers, lignin, in nature. Both peroxidases and laccases exhibit low substrate specificity and produce organic radicals through one-electron abstraction reactions. In many cases these enzymes require low molecular weight co-substrates to mediate oxidation to the more recalcitrant parts of lignin polymer. In the first study of this thesis, various types of lignin model compounds were investigated both as mediators and as final targets to screen their effect and potential in laccase-catalyzed oxidation reactions. Especially acetosyringone and methyl syringate were found to mediate the laccase-catalyzed oxidation even with low-redox potential laccase that generally shows only fair oxidation activity on lignocellulosic oxidation. Secondly, in two of the articles, the mediating rate and effectivity were enhanced by using high redox potential laccases and suitable reaction conditions to retain the stability of the mediator. Oxidation potentials, pH and chemical stability of the mediator were found to affect the oxidation efficiency. These simple phenolics were, for the first time, also proved to mediate the MnP-catalyzed reactions of non-phenolic compounds. The problematic stability of peroxidases was circumvented by utilizing reaction system with no external addition of hydrogen peroxide. Finally, to prove the effect on polymer material a new reaction set-up was developed for oxidation of synthetic lignin (DHP, dehydrogenation polymer) and the structural analysis of the oxidized polymers showed clear modifications in the polymer outcome. In all cases, mediated reactions gave selective α−oxidation products of benzylic alcohol moieties, the prevailing structural moiety in lignin. These oxidations provide valuable intermediates for further treatments in various lignin valorization processes. In the near future, this oxidative laccase-mediator oxidative system will be explored in an EU Horizon 2020 project for valorization of biorefinery lignin by combination of biotechnical and chemical means to added-value products such as marine biofuel, engine octane boosters and valuable low-molecular weight chemicals.

Keywords: lignin, lignin model compounds, dehydrogenation polymer (DHP), lignocellulose, mediators, oxidation, radical reactions, hydrogen atom transfer, oxidoreductases, laccase, manganese peroxidase, versatile peroxidase

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LIST OF ABBREVIATIONS AND DEFINITIONS

AAO Aryl-alcohol oxidases
AAD aryl-alcohol dehydrogenases
ABTS 2,20 -azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
β-O-4 lignin arylglycerol-β-aryl ether structure
β-5 lignin phenylcoumaran structure
β-β lignin resinol structure
β-1 lignin diphenylethane structure
5-5' lignin biphenyl structure
4-O-5' lignin diaryl ether structure
5-5'-O-4 lignin dibenzodioxocine structure
CAZy carbohydrate degrading hydrolase
CDH cellobiose dehydrogenases
CEL cellulotic enzyme lignin
CO_2 carbon dioxide
DHP dehydrogenation polymer
EMAL enzymatic mild acidolysis lignin
ET electron transfer
GLOX glyoxal oxidases
3HAA 3-hydroxyanthranilic acid
HAT hydrogen atom transfer
HBT 1-hydroxybenzotriazole
H_2O_2 hydrogen peroxide
HPI N-hydroxyphtalimide
kDa MW unit 1000 Da
LCC lignin carbohydrate complexes
LiP lignin peroxidase
LMS laccase mediator system
LPMO lytic polysaccharide mono oxygenase
LRET long range electron transfer
MnP manganese peroxidase
M_N number average molecular weight
M_W weight average molecular weight
MW molecular weight
MWL milled wood lignin
NHA N-hydroxyacetamide
NHE normal hydrogen electrode
NMR nuclear magnetic resonance spectroscopy
O_2 oxygen
PAH polycyclic aromatic hydrocarbons
PDI = M_W/M_N polydispersity index
QR quinone reductases
S1, S2, S3 secondary cell wall layers
SEC size exclusion chromatography
TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxyl free radical
VP versatile peroxidase

LIST OF ORIGINAL PUBLICATIONS

The present doctoral thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Nousiainen P., Maijala P., Hatakka A., Martínez A.T. and Sipilä J. (2009) Syringyl-type simple plant phenolics as mediating oxidants in laccase catalysed degradation of lignocellulosic materials: model compound studies. Holzforschung 63(6), 699-704.

II. Uzan E., Nousiainen P., Balland V., Sipila, J., Piumi F., Navarro D., Asther M., Record E. and Lomascolo, A. (2010) High redox potential laccases from the ligninolytic fungi *Pycnoporus coccineus* and *P. sanguineus* suitable for white biotechnology: from gene cloning to enzyme characterization and applications. J. Appl. Microbiol., 108(6), 2199-2213.

III. Nousiainen, P., Kontro, J., Maijala, P., Uzan, E., Hatakka, A., Lomascolo, A., Sipilä, J. (2013) Lignin model compound studies to elucidate the effect of 'natural' mediators on oxidoreductase catalyzed degradation of lignocellulosic materials. In Functional Materials from Renewable Sources, ACS Symposium Series, 1107, Liebner, F. & Rosenau, T. (eds.), pp. 229-242.

IV. Nousiainen P., Kontro J., Manner H., Hatakka A., Sipilä J. (2014) Phenolic mediators enhance the manganese peroxidase catalyzed oxidation of recalcitrant lignin model compounds and synthetic lignin. Fungal Genetics and Biology special issue on Plant biomass degradation by fungi, 72, 137-149.

The author has contribution to following papers not included in this thesis:

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Maijala, P., Mattinen, M.L., Nousiainen, P., Kontro, J., Asikkala, J., Sipilä, J., Viikari, L. (2011) Action of fungal laccases on lignin model compounds in organic solvents. Comparison of various laccases in the oxidation of lignin model compounds in organic solvents. J. Mol. Catal. B: Enzymatic. 76, p. 59-67.

Mattinen M.L., Maijala, P., Nousiainen, P., Kontro, J., Sipilä, J., Tamminen, T., Viikari, L. (2011) Oxidation of lignans and lignin model compounds by laccase in aqueous solvent systems. J. Mol. Catal. B: Enzymatic. 72, 3-4, p. 122-129.

Saastamoinen, P., Mattinen, M.L., Suurnäkki, A., Hippi, U., Nousiainen, P., Sipilä, J., Lille, M., Pere, J. (2012) Laccase aided modification of nanofibrillated cellulose with dodecyl gallate. Bioresources, 7(4), 5749-5770.

Areskogh, D., Li, J., Nousiainen, P., Gellerstedt, G., Sipilä, J., Henriksson, G. (2010) Oxidative polymerization of models for phenolic lignin end-groups by laccase. *Holzforschung,* 64, 21-34

Areskogh, D., Nousiainen, P., Li, J., Gellerstedt, G., Sipilä, J., Henriksson, G. (2010) Sulfonation of phenolic end-groups in lignin. Direct laccase initiated reaction towards cross-linking. *Industrial Biotechnology* 6(1): 50-59.

Camarero, S., Canas, A. I., Nousiainen, P., Record, E., Lomascolo, A., Martínez, M. J., and Martínez, A. T. (2008) p-Hydroxycinnamic Acids as Natural Mediators for Laccase Oxidation of Recalcitrant Compounds. *Environ. Sci. Technol. 42*, 6703–6709.

Contribution of the author on publications

I Paula Nousiainen planned the experiments, carried out the laboratory work and analyzed and interpreted the results and wrote the article with contribution of the other authors.

II Paula Nousiainen planned the lignin model compound experiments, did the laboratory work and analyzed and interpreted the results.

III Paula Nousiainen planned the experiments, carried out the laboratory work and analyzed and interpreted the results and wrote the book chapter with contribution of the other authors.

IV Paula Nousiainen planned the experiments, carried out the laboratory work together with Jussi Kontro and Helmiina Manner, apart from the solvent stability testing of the peroxidases, which was carried out in Department of Food and Environmental Sciences, University of Helsinki. FM Sami Hirvonen ran the SEC analyses in the Laboratory of Polymer Chemistry, University of Helsinki. Paula Nousiainen analyzed and interpreted the results and wrote the article with contribution of the other authors.

1. INTRODUCTION

One of the most important challenges in modern industrial development deals with eco-efficient and eco-friendly applications of plant biomass as feedstock for fuels, chemicals and energy, to reduce our dependence on fossil-originated materials (Ragauskas *et al.* 2006, Clark 2007, Bozell 2008). Thanks to the rapid advancement of modern biotechnology and genetic engineering, enzymes are studied as economically feasible, and environmentally friendly industrial biocatalysts to exert the required chemical conversions in future biorefineries (Xu 2005, Martínez *et al.* 2009, Wohlgemuth 2010).

Plant biomass is a complex group of biomolecules and polymers, and is generally considered to consist of a mixture of chemically relatively labile (sugars, starch, hemicellulose, and cellulose) and more recalcitrant (lignin, suberin, and cutin) compounds (Aneja *et al.* 2006, Theuerl and Buscot 2010). Cellulose and starch are already thoroughly exploited and found in several technical applications in the chemical industry. In the case of hemicelluloses and lignin this process has been much slower. Although produced in large quantities e.g. in wood pulping, so far these polymers have mostly been utilized as a source of energy but not further refined into chemical feedstock. In vascular plants these polymers make approximately half of the dry weight of the cell walls and occur there roughly in equal proportions. Based on their abundance alone they certainly will have an important role in biorefining technologies. The particular technical interest in lignin is that - contrary to hemicellulose - lignin is a polyphenol and a primary source of aromatic compounds in nature. Lignin has a potential role as raw-material for example in the production of renewable fuels, adhesives, binders, anti-oxidants, bulk chemicals and carbon fibers (Rex 2010, Zakzeski *et al.* 2010) but its industrial utilization still awaits breakthrough.

The significant disadvantage in utilization of lignin is its heterogenic, polydisperse and complex three-dimensional structure and location in the cell wall interwoven with the carbohydrate matrix (Adler 1977, Ralph *et al.* 2004). The macromolecule is formed through enzyme initiated oxidative radical polymerization and according to their composition lignins can be divided into softwood, hardwood and grass lignins. Monolignols are mainly linked by various ether and C-C bonds, but the actual mechanism of polymerization in cell tissues has not been completely established (Boerjan *et al.* 2003, Ralph *et al.* 2004, Vanholme *et al.* 2008). The molecular weight, structural features and amount of lignin vary between different plant species and depend on how the lignin material is isolated. Despite the combination of modern analytical techniques used for structural elucidation, the exact structure of native and especially processed lignins remains undefined. Because of these difficulties in separation and analysis of lignin, simpler model compounds are commonly applied.

Oxidative enzymes such as peroxidases and laccases intimately participate in lignin biosynthesis but they are also key elements in lignin biodegradation and therefore a potential source for industrial ligninolytic biocatalysts (Xu 2005, Martínez *et al.* 2009). Wood and litter degrading basidiomycetous white-rot fungi are the only organisms able to degrade lignin efficiently (Hatakka and Hammel 2010). They secrete extracellular ligninolytic enzymes (lignin-degrading oxidative enzymes) including lignin peroxidases (LiPs), manganese peroxidases (MnPs), versatile peroxidases (VPs) and laccases (p-diphenol:oxygen oxidoreductases). These enzymes show the ability to degrade and even mineralize lignin to carbon dioxide and water through intermediate formation of organic radicals and diffusible, oxidative mediators (Kirk and Farrell 1987, Tuor *et al.* 1995, Hatakka and Hammel 2010).

Most oxidative biotechnical applications have focused on laccases that can already be produced on an industrial scale and can be tailored by modern biotechnological means. Ligninolytic basidiomycetous fungi produce laccases with the highest redox potentials, but often these wild-type enzymes do not meet the demands of industrial applications in terms of stability, thermotolerance and enzyme production. Laccases from ascomycetous fungi on the other hand have been more easily tailored to industrial purposes. For the generation of tailor-made laccases the preliminary step includes screening of the prominent enzymes from biodiversity with the purification and characterization of the proteins, followed by subsequent gene encoding by molecular breeding (Otterbein *et al.* 2000, Martínez *et al.* 2005, Ayala *et al.* 2008, Uzan *et al.* 2010).

Laccases belong to a superfamily of multicopper oxidases forming therein a phylogenetically divergent group of so called "blue oxidases". They are extracellular glycoproteins of about 500 amino acid in length (typically 55–85 kDa) and contain four copper atoms distributed in one mononuclear (T1) and one trinuclear (T2/T3) domain in the enzymes' active center. Laccases are capable of catalyzing oxidation of a wide range of organic substrates, such as phenols and polyphenols, aromatic amines, thiols and heterocyclic compounds and even inorganic salts (Xu 1996). The wide substrate spectrum, the stability of laccases towards chemical solvent systems, their thermostability and the lack of substrate inhibition have made laccases feasible for industrial applications as reviewed by Call and Mücke (1997), Kunamneni *et al.* (2008), Upadhyay *et al.* (2015).

Ligninolytic extracellular peroxidases have the advantage of having higher redox potentials and thus also a broader substrate range than laccases. Ligninolytic peroxidases contain a heme prostethic group (iron(III) protoporphyrin IX) in their active center. LiP has high enough redox potential to directly oxidize non-phenolic lignin subunits by Cα-Cβ cleavage in β-O-4 ether structures. MnPs, on the other hand, specifically oxidize Mn(II) to reactive Mn(III), which acts as a diffusible mediator. Mn(III) requires organic acids, like oxalic or malonic acid, to provide stabilization by chelation, and it oxidizes phenolic compounds directly or, in turn oxidizes a second mediator e.g. thiols or unsaturated fatty acids and their derivatives for the degradation of non-phenolic compounds (Ruiz-Dueñas *et al.* 1999, Kapich *et al.* 2005). As a third class, VPs are hybrids of lignin peroxidase and manganese peroxidase with a bifunctional characteristic (Hofrichter 2002). Drawbacks of ligninolytic peroxidases are their limited commercial availability and problematic activity. They require hydrogen peroxide but are also inhibited by an excess of peroxide (Taboada-Puig *et al.* 2011). This means that the control of constant peroxide production is essential to carry out effective oxidation (Timofeevski *et al.* 1998, Bockle *et al.* 1999, Valderrama *et al.* 2002).

Both peroxidases and laccases catalyze their substrate oxidation by a mechanism involving free radicals, and showing low substrate specificity. These reactions produce organic radicals through one electron abstractions predominantly from phenolic substrates. Such radicals (in case of LiP also radical cations) can then undergo various spontaneous follow-up reactions independent of enzyme activity, like polymerization via radical coupling, aromatic ring cleavage, or breaking C-C bonds (Wong 2009). Laccases and MnP/Mn(III)-chelates, owing to their comparatively low redox potentials are, as such, capable to oxidize only the phenolic structures (Martínez *et al.* 2005). However, they are the predominant catalysts, for example, in lignin biodegradation.

The lively research on laccases and laccase mediators has revealed that combined with a co-catalyst, so called mediators or enhancers, the range of laccase substrates may be

considerably extended to more recalcitrant ones e.g. non-phenolic lignin moieties and environmental xenobiotics. As a consequence, potential biotechnical applications of laccases now include a wide spectrum of applications such as decolorization and detoxification of effluents from the textile and dyestuff industries, pulp and paper industries, olive mill wastes and coffee pulp as well as the degradation and detoxification of recalcitrant wastewater pollutants (chlorophenols, endocrine disrupting compounds (EDCs), polycyclic aromatic hydrocarbons (PAHs), pesticides and insecticides) and immobilization of soil pollutants as well as various other applications, such as wood fiber modification, biosensors and organic synthesis (Xu 2005, Wells *et al.* 2006, Widsten and Kandelbauer 2008, Tuomela and Hatakka 2011).

Most of the mediators have been synthetic compounds based on nitrogen heterocyclics such as TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical) or HBT (1-hydroxybenztriazole). The mediator–substrate oxidation has been reported to occur either via an ionic mechanism e.g. TEMPO and its analogues, or via two radical mechanisms. The radical mechanisms proceed via electron transfer (ET) with e.g. ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) or hydrogen atom abstraction (HAT) with e.g. HBT (Fabbrini *et al.* 2002, Riva 2006). In the latter HAT oxidation, the primary target for oxidation is the benzylic position of arylpropane units to produce the corresponding α−carbonyl structures prone to various degradation reactions. The exploitation of laccase mediators in industrial processes has been limited, since they are expensive in amounts required in oxidation processes and they are mostly harmful to the environment as chemicals. Recently, it has been found that some natural substances derived from syringyl-type plant phenolics are good substrates for laccases, offering sufficiently long life-times and the ability to mediate the oxidation in substances which are recalcitrant towards oxidation by laccases (Camarero, Ibarra, Martínez, and Martinez 2005, Kunamneni *et al.* 2007, Camarero *et al.* 2008, Canas and Camarero 2010). Simple syringyl-type phenolics are potentially cheap and readily available from e.g. downstreams of hardwood pulping industry wastes (Camarero, Ibarra, Martínez, and Martinez 2005, Canas and Camarero 2010). Natural phenolics potentially react further in laccase-catalyzed reactions resulting in more environmentally sustainable processes.

The aim of this PhD-work was to screen the applicability of laccase–natural mediator systems for the industrial lignin-processing sector. For this purpose, the oxidation of different non-phenolic lignin model compounds (representing the most common lignin structural units) was elucidated in reaction systems that consisted of natural syringyl-type phenols as a mediator and different oxidoreductases as the oxidation catalysts. It was found that these syringyl-type phenols were able to mediate even the low-redox potential ascomycete laccase *Myceliophthora thermophila*-catalyzed oxidation of lignin model substrates of higher oxidation potential, and also in small concentrations (**Publication I**). The oxidation capacity could be enhanced by using high-redox potential basidiomycete laccases from the *Pycnoporus coccineus* and *Pycnoporus sanguineus* species (**Publication II**) which also showed marked differences in their mediated oxidation capacities. Comparative study with high- and low-redox potential laccases from the *Pycnoporus* and *M. thermophila* species revealed that the reaction conditions had a remarkable effect on oxidation capacities and that the choice of pH range in the reaction should be made according the mediator used, not according to the optimum pH of the laccase (**Publication III**). The results inspired us to evaluate the potential of these natural mediators also with

peroxidases that use Mn^{2+}/Mn^{3+} redox-systems as oxidants (**Publication III and IV**). Two peroxidases from white-rot fungi, MnP from *Phlebia* sp. b19 and VP from *Bjerkandera adusta* were used in MnP reaction conditions (in the presence of Mn^{2+} at pH 4.5-5.5) and syringyl-type phenols as mediators. The natural-type mediators were found to enhance also the oxidation reactions by MnP and VP. Here also a new type of reaction system was developed, based on the oxidation of synthetic lignin (DHP) in cellulose slurry, acting as a 'co-solvent' and a mimic to natural lignocellulose, and this cellulose bound DHP material was then treated as a substrate in an enzymatic-mediator –system with isolation by simple solvent extraction to give oxidized low and high molecular weight fractions for further analysis. The reactions were also found to proceed steadily without any external addition of hydrogen peroxide into the reaction; a crucial drawback of the biotechnical use of peroxidases is often that an excess of peroxide in the system inactivates the catalyst. The system generated peroxides *in situ*, as the degradation products of organic diacids formed a source of the reducing oxidants for peroxidases in order to continue the reaction for several days (**Publication IV**).

2. BACKGROUND

2.1 EVOLUTION AND RELEVANCE OF LIGNIN FOR PLANTS AND ECOSYSTEM

Plant biology has gained revolutionary advancements in plant genome sequencing projects and functional genomics which have facilitated the phylogenetic analysis of the genes in order to reveal the evolutionary history of plants. It has been suggested that the evolution of the phenylpropanoid secondary metabolism 450-500 million years ago (during the Late Ordovician period) which brought about the production of lignin by the early vascular plants, has been one of the critical adaptations that allowed plants to grow upwardly and to colonize the Earth's terrestrial ecosystems (Weng and Chapple 2010, Labeeuw *et al.* 2015). The development of tracheids with lignified water conducting tissues allowed the vascular plants to grow taller and survive drier conditions than the earlier bryophytic plants (Weng and Chapple 2010, Oinonen *et al.* 2015). As the plants themselves do not possess a mechanism to degrade lignin, the atmospheric carbon (carbon dioxide) fixed in lignin biosynthesis was not recoverable and lignin became a major carbon sink in plants. The development of the terrestrial plants contributed to the formation of lignin-rich soil biomass that was buried and fossilized, thus forming a coal and fossil fuel reserve during the Carboniferous period 360-300 million years ago. This resulted also in significant changes in the Earth's climate, intensifying a drop in atmospheric CO_2 levels and a corresponding increase in O_2 levels (from 10% up to 35%) with considerable cooling during the late Coniferous period.

Some early microbes adapted to the oxidizing atmosphere by either modifying parts of their metabolism or by forming symbiotic associations that permitted the utilization of lignin-derived aromatic molecules as carbon sources coupled with a reduction of O_2 back to H_2O. Floudas et al (2012) suggested that the evolution of a completely new type of fungi with the ability to degrade lignin coincided with the end of the formation of the lignin-rich geologic coal deposits that drastically ceased around 300 million years ago (Floudas *et al.* 2012). These fungi used oxidative enzymes to produce radicals that could fragment and break up the recalcitrant lignin and finally convert it back to carbon dioxide.

Utilizing the fossil deposits facilitated the development of the modern industrialism from the 19th century that we are dependent on nowadays. However, the anthropogenic combustion of these more than 300-year-old fossil coal deposits back to atmospheric CO_2, the well-known greenhouse gas, has increased its amount by 35% (from 280 to 400 ppm) during the last century compared to pre-industrial time (Raupach and Canadell 2010). This has changed the dynamic climate balance that is interactively dependent on the atmosphere, land surface, polar ice, oceans and plant ecosystems. The global warming has forced us to strive to find alternative ways to produce energy and fuels from lignocellulosic biomass (Balat 2011, Gupta and Tuohy 2013). Because of its high abundance and availability, lignocellulose is considered as the only sustainable organic resource that can be converted to fuel, energy and diverse value-added chemicals and products.

2.2. THE STRUCTURE AND RECALCITRANCE OF THE PLANT CELL WALL

Land plants fix atmospheric carbon dioxide, CO_2, via photosynthesis into lignocellulosic biomass. The global annual formation of biomass has been estimated at 170-200 x 10^9 tons (Pauly and Keegstra 2008) and it forms the most abundant renewable resource on Earth. The plant cell wall of vascular plants and woods is composed of three major polymeric components: cellulose, hemicellulose and lignin. In addition to these it contains some minor components such as extractives (solvent-soluble non-volatile compounds like lignans, terpens, fatty acids, resins, waxes, pectin, chlorophyll etc.), proteins and inorganic elements usually referred to as the ash contents (P, K, Ca, and Mg etc.) (Cornwell *et al.* 2009, Sannigrahi and Ragauskas 2013). The proportion of the components depends on the plant type. Softwood and hardwood cellulose content is in the range of 40–42%, hemicelluloses roughly 20–30% and lignin 25–32%, whereas agricultural residues contain 30–40% cellulose, 20–30% hemicelluloses and 15–30% lignin. The content of other components varies between 5-10% (Bornscheuer *et al.* 2014).

The plant cell wall is a tough extracellular matrix that encloses each cell in a plant. It has a "skeletal" role in supporting the structure of the plant, a protective role for each cell individually, and a transport role by forming channels for the movement of fluid and nutrients in the plant (Alberts *et al.* 2002, Pauly and Keegstra 2008, Keegstra 2010). The walls of neighboring plant cells are cemented together to form the intact plant and they differentiate from an animal cell wall matrix by being thicker, stronger, and more rigid. The plant cell wall consists of three types of layers (Figure 1). The middle lamella forms the outer wall of the plant cell and is shared by adjacent cells. The middle lamella is formed of mainly pectins, lignin and proteins and it ensures the adhesion between the cells. The primary cell wall, which is formed after the middle lamella to accommodate subsequent cell growth consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectins, hemicellulose and glycoproteins. The primary cell walls are tough, but also thin and extensible. The secondary cell wall is formed by depositing new layers inner to the primary wall after the cell enlargement is completed and it is composed of cellulose, hemicellulose and lignin. The secondary wall is extremely rigid and often layered (S1, S2, S3) and provides compression strength for the plant. The cellulose microfibrils may be aligned irregularly (as in the primary cell wall), or at a particular angle to the cell axis (as in layer S1, S2, and S3). Lignification constitutes the last stage of cell division, expansion, and elongation before cell death. The lignification mechanism is still not completely understood.

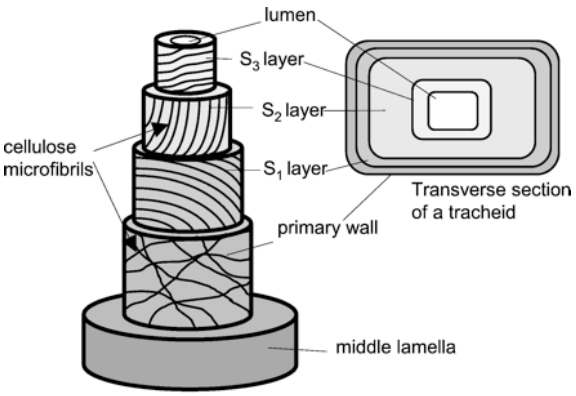


Figure 1. Three-dimensional structure of the secondary cell wall of a tracheid (xylem cell). The cell wall is divided into different layers, each layer having its own particular arrangement of cellulose microfibrils, which determine the mechanical and physical properties of the wood in that cell. Reprinted with permission from Plomion *et al.* (2001). Copyright 2001 the American Society of Plant Biologists.

Lignocellulosic biomass is the only sustainable organic resource that can be converted to both energy and various value-added products. The chemical composition of biomass and the way in which different cell wall components interact between each other, i.e. the inherent resistance of the plant cell wall, determines its suitability as an economically viable feedstock and the optimum pathway for its conversion to fuel and materials, but it determines also the substrate quality for microbial decomposers (Fukuda *et al.* 2009, Sánchez 2009). The effective use of wood and plants for chemical pulping processes and as bioenergy feedstock is in most cases dependent on the cell wall recalcitrance and especially on the extent of lignification of the cell wall.

2.2.1 STRUCTURE OF LIGNIN

Lignin is an important structural component of water-conducting cells of wood and vascular tissues. Lignin is the second most abundant polymer with 15–35% constitution of lignocellulosic biomass dry-weight and the most abundant aromatic polymer in nature. It possesses the highest specific energy content (25-26.5 MJ/kg) of all biomass fractions. Lignin is an amorphous, complex three-dimensional polyphenolic heteropolymer composed of phenylpropane units interconnected by various stable carbon-carbon and ether linkages (Ralph *et al.* 2004, Brunow and Lundqvist 2010). The role of lignin is to provide mechanical strength for plant cell walls by binding the fibers together through an intermolecular network, giving it structural rigidity to grow upwards. As a hydrophobic polymer, it decreases the permeation of water through the cell wall and strengthens the water-conducting cells for long-distance water and nutrient transport for the plant. Lignin also provides the plant's resistance against degrading microbes, enzymes and chemicals.
In lignin biosynthesis, the cinnamic acids building blocks are formed through the general phenolic metabolism pathways. The shikimic acid pathway with synthesis of phenylalanine is followed by the phenylpropanoid metabolism to produce the activated cinnamic acid derivatives for lignin synthesis. The primary three phenylalanine-derived *p*-

hydroxycinnamyl alcohol monomeric precursors, monolignols, are *p*-coumaryl alcohol (4-hydroxy-cinnamyl alcohol), coniferyl alcohol, (4-hydroxy-3-methoxycinnamyl alcohol) and sinapyl alcohol (4-hydroxy-3,5-dimethoxycinnamyl alcohol) shown in Figure 2. (Adler 1977, Ralph *et al.* 2004, Vanholme *et al.* 2010).

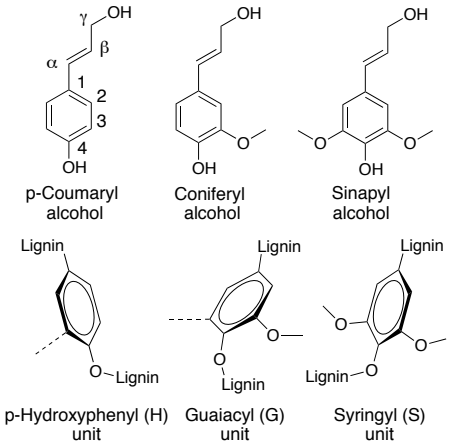


Figure 2. *p*-Hydroxycinnamyl alcohol monomers and the corresponding structural units in lignin indicated as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units.

The monolignols are synthesized in the cytoplasm, after which they are transported across the plasma membrane to the cell wall where they undergo one electron oxidation by oxidizing enzymes, the plant peroxidases and laccases. Consequently, the formed electron-delocalized radicals with unpaired electron density at their 1-, 3-, O-4-, 5-, and β-positions undergo spontaneous radical coupling with each other (Figure 3). The coupling is generally favored at the β-position of the reacting monolignols, which results in the formation of arylglycerol-β-aryl ethers (β-O-4), phenylcoumarans (β-5), resinols (β-β), diphenylethane dimers (β-1), and spirodienones. When the dilignols couple further, the aryl positions 4 and 5 are favored, giving biphenyls (5-5'), dibenzodioxocines (5-5'-O-4) and 4-O-5' diaryl ethers. Various studies on isolated lignins have shown that the dibenzodioxocin structure is most probably the main branching point in softwood lignin with comparatively high abundance (Karhunen *et al.* 1995, Heikkinen *et al.* 2003, Kukkola *et al.* 2003). Only a small proportion of the phenolic hydroxyl groups are free (approximately 10 - 20%) since most are occupied in linkages to neighboring phenylpropane units (Adler 1977). It is generally accepted that lignin is a racemic and non-repeating polymer forming an irregular noncrystalline network (Ralph *et al.* 2004). Different structures produced by oxidative coupling reactions are presented in Figure 3.

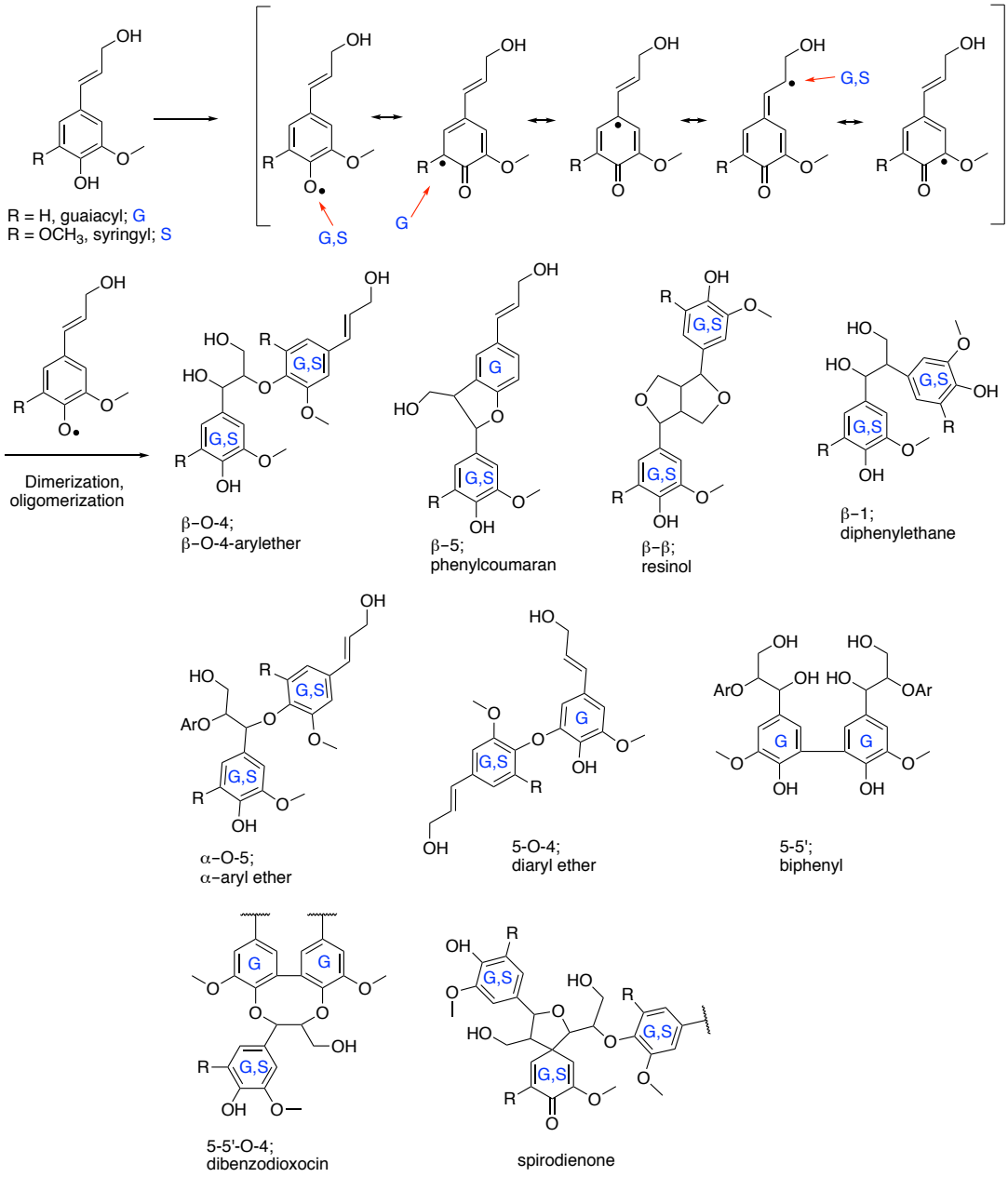


Figure 3. The reaction path starting from *p*-hydroxycinnamyl alcohol monomers, coniferyl (R=H) or sinapyl alcohol (R=OCH_3), and advancing onto different type of dimers or oligomers. The red arrows show the reaction site for the end-wise oxidative polymerization.

A typical structural unit abundance in different types of lignins is presented in Table 1. The values are determined by different chemical or spectroscopic methods (Adler 1977, Zakzeski *et al.* 2010, Munk *et al.* 2015).

Table 1. Typical structural unit frequency in different types of isolated native lignins, based on (Adler 1977, Zakzeski *et al.* 2010, Munk *et al.* 2015).

	Amount per 100 C9 units		
Linkage	Grass	Softwood	Hardwood
β-O-4 (β–arylether)	69-94	36-50	60-80
β-5 (phenylcoumaran)	11-14	9-13	3-11
β-β (resinol)	4-15	2-6	3-12
β -1 (diphenylethane)	low	1-9	1-7
5,5'-O-4 (dibenzodioxocine)	3-4	5-7	0-2
spirodienone	3-5	1-2	3-5
5-5'(biphenyl)	-	19-27	3-9
4-O-5' (diaryl ether)	-	3.5-8	6-9
Phenolic-OH		15-20<	10-15

The molecular weights (MW) of lignin and especially native lignin have been difficult to determine since the isolation of lignin from cell walls usually affects also its structure. Some estimations of lignin MW and the polydispersity are presented in Table 2 together with their extraction yields based on the Klason lignin content of the ground wood meal (Tolbert *et al.* 2014).

Table 2. Examples of the molecular weights of different isolated lignins based on (Tolbert *et al.* 2014).

Biomass	% yield	M_N	M_W	PDI
Softwood *Norway spruce*				
MWL	11.4	6400	23500	3.7
CEL	23.4	9450	53850	5.7
EMAL	44.5	8850	78400	8.8
Hardwood *E. globulus*				
MWL	34.0	2600	6700	2.6
CEL	32.5	5500	17200	3.1
EMAL	63.7	8700	32000	3.7

Norway spruce, *Picea abies; E. globulus, Eucalyptus globulus;* MWL milled wood lignin; CEL cellulotic enzyme lignin; EMAL enzymatic mild acidolysis lignin; M_W weight average molecular weight; M_N number average molecular weight; PDI=M_W/M_N polydispersity index.

The resulting polymer is a heterogenous, polydisperse and complex three-dimensional structure (tentatively illustrated in Figure 4) and it is assumed to exist as a web shaped rather than a linear or branched polymer. It is both physically entangled and covalently associated with the carbohydrate matrix (cellulose and hemicelluloses) providing further stiffness to the matrix. The lignin carbohydrate complexes (LCC) have been shown to exist as a constituent

part of lignin in wood (Lawoko *et al.* 2005, Oinonen *et al.* 2015). The most common linkages seem to be ether linkages to benzylic α–carbon or phenyl glucosides, but also α- and γ -esters are important especially in grasses (Oinonen *et al.* 2015).

Gymnosperm woods (softwood) contain almost exclusively (95%) guaiacyl lignin based on coniferyl alcohol whereas angiosperms (hardwood) also contain (around 50%) syringyl units derived from sinapyl alcohol. All groups, but especially monocots (grass lignin), also contain small amounts (2-5%) of *p*-hydroxyphenyl units (Ralph *et al.* 2004, Grabber 2005, Brunow and Lundqvist 2010). In addition to these building blocks, also some other non-conventional γ-acylated (e.g. acetylated, *p*-coumaroylated, *p*-hydroxybenzoylated) hydroxycinnamic acid precursors contribute in lignin biosynthesis in some plant species (del Río *et al.* 2007). In addition to differences in lignin composition among plant species and taxa, the composition can also differ within cell types and even at the level of individual cell wall layers.

The exact structure of lignin has been very difficult to resolve and depends on the origin of the lignin sample and the isolation technique. Often, different types of lignin model compounds are used in order to obtain insight into the mechanistic details of the reactions in lignin conversion. Specifically, the use of lignin model compounds allows the evaluation of the reactivity of a specific type of linkage represented by the model compound.

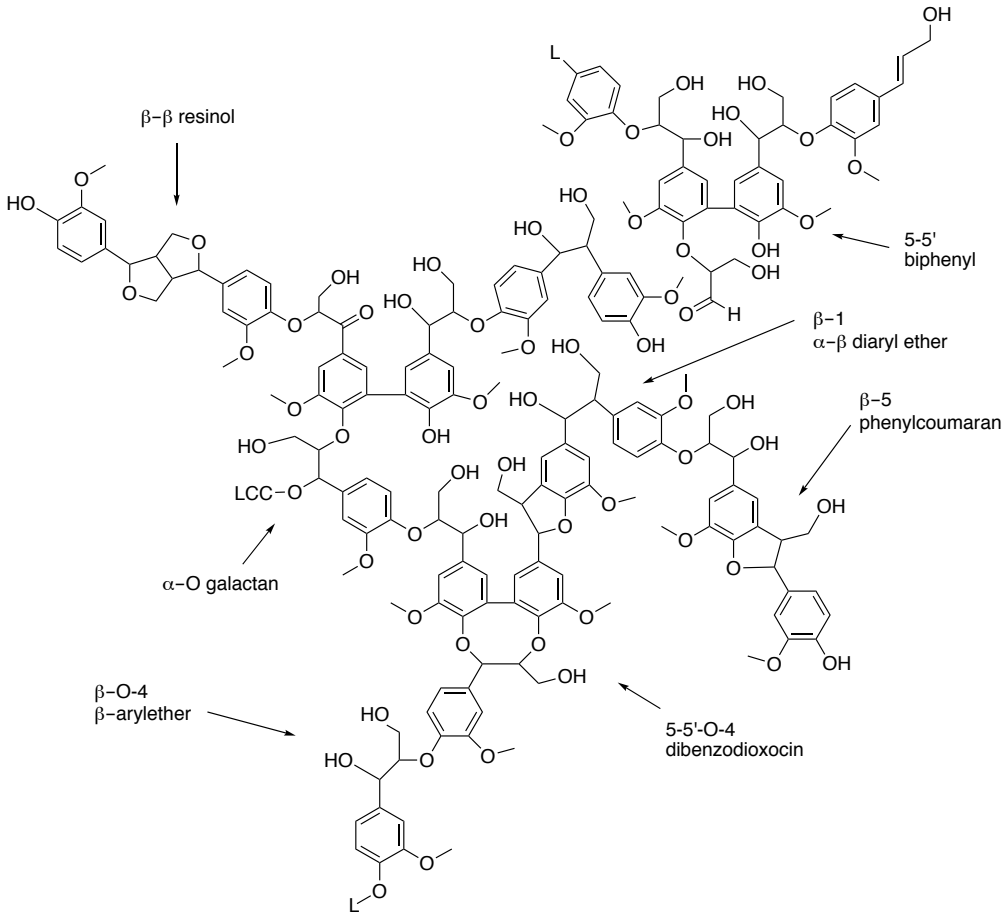


Figure 4. The tentative representation of lignin structure of coniferins with different structural patterns present in polymer modified from Brunow *et al.* (1998).

The complex nature and recalcitrance of lignin implies that it is biologically and chemically difficult to degrade.

2.2.2 STRUCTURE OF CELL WALL CARBOHYDRATES CELLULOSE AND HEMICELLULOSE

Cellulose is the most abundant carbohydrate (30-50% of dry biomass) on earth, and it is a homopolysaccharide consisting of D-glucose units that are linked by β-1,4-glycosidic bonds. Each glucose unit is rotated 180° relative to its two neighbors, thus forming linear chains with 800 to 10000 glucose units in plant fibers (Klemm *et al.* 2005). Individual cellulose chains are associated to each other by hydrogen bonds and Van der Waals forces to form a highly crystalline structure called microfibrils. The microfibrils have characteristic orientations (helix angles), which differ as a function of the cell wall layer and according to the plant type. Cellulose molecules consist of highly crystalline regions in the microfibrils separated by less ordered amorphous regions, and depending on the source the crystallinity varies from 50% to 90%. The crystalline nature of cellulose makes it relatively resistant to enzymatic and chemical degradation (Pérez *et al.* 2002).
Hemicelluloses are a group of heteropolysaccharides (20-30% of dry biomass) with significantly lower molecular weight than cellulose. They consist of pentoses (D-xylose, L-arabinose), hexoses (D-glucose, D-mannose, D-galactose), and sugar acids (4-O-methyl-glucuronic and D-galacturonic acid). These monomeric sugars are linked together by β-1,4- and occasionally by β-1,3-glycosidic bonds to form the backbone of hemicelluloses with 50-300 glycan units. The major hemicellulose component in hardwood is glucuronoxylan, whereas glucomannan is predominant in softwood (Saha 2003). The most important biological function of hemicelluloses is reinforcing the cell wall structure by cross-linking cellulose microfibrils and lignin via direct interactions. Since hemicelluloses are branched with short lateral chains, they do not form crystalline structures like cellulose and therefore their hydrolysis can be performed relatively easily. However, because of the hemicellulose heterogenic nature their enzymatic degradation requires a variety of respective degrading-enzymes (Pérez *et al.* 2002, Saha 2003).

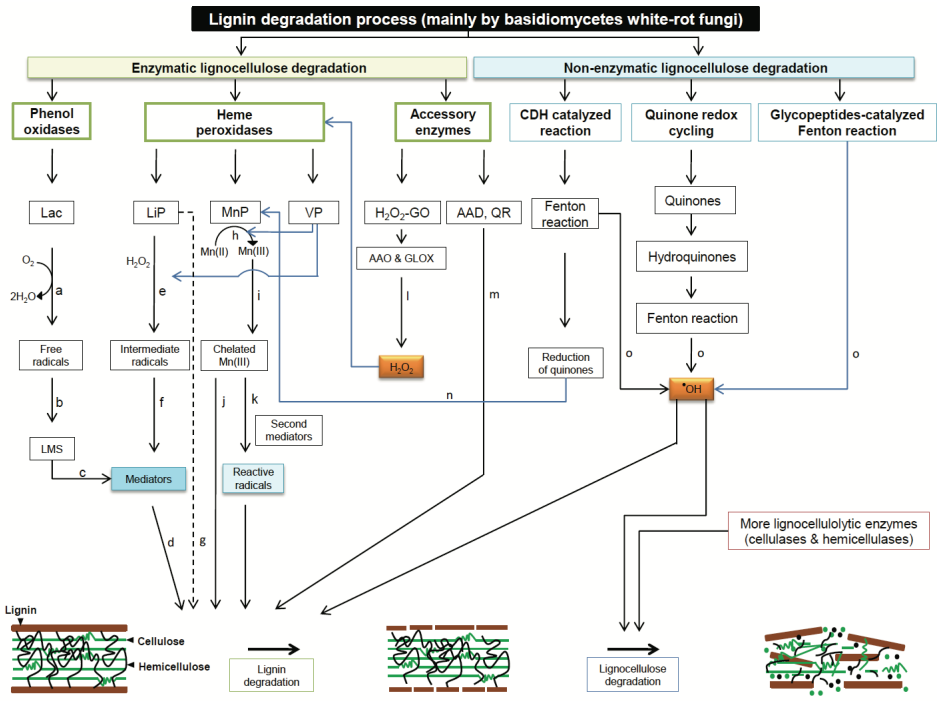
2.3 PLANT BIOMASS DEGRADING FUNGI AND THEIR ENZYMES

2.3.1 FUNGAL DEGRADATION SYSTEMS

In the secondary plant cell walls of wood, the lignin matrix that surrounds the cellulosic components acts as a barrier to wood decay, preventing the access of extracellular enzymes to the more readily degradable cellulose and hemicelluloses. Contrary to carbohydrates, lignin is non-hydrolysable under biological conditions (Boerjan 2005, Hammel and Cullen 2008) and resists attacks by most microorganisms. Anaerobic processes for example are not assumed to effectively attack the aromatic rings and the aerobic breakdown of lignin is slow whereas the polysaccharide components are more readily attacked (Benner *et al.* 1984, Kirk and Farrell 1987). Specific basidiomycete fungi can enzymatically attack all the polymers in lignocellulose and in this way enable the fast nutrient recycling from degraded wood back to new plant growth and even mineralize it to carbon dioxide and water. In general, nature uses five basic chemistries, oxidation, hydrolysis, dehydration, reduction and free radical reactions to convert lignocellulosic material, and the degradation of wood is an irreversible process (Nilsson and Rowell 2012). These degrading enzymes together with information on enzymes with auxiliary activities, can be divided into multiple families and subfamilies by

using constantly collected information on their sequence, catalytic mechanism and enzymatic specificity. During biomass conversion by fungi, many of the lignin- and carbohydrate-active redox enzymes are expressed simultaneously with hydrolytic enzymes, which points to a possible interplay between these enzyme systems (Hatakka 2001, Hatakka and Hammel 2010, Kües 2015, Kuuskeri *et al.* 2016). Fungal decay in natural environment needs appropriate moisture and temperature conditions to proceed but also proper soil pH and nutrient composition, and these demands vary depending on the fungal species (Kües 2015).

Due to the complexity, random composition and variety of the lignin chemical linkages, together with their hydrophobic structure, lignin degradation requires the synergistic action of several enzymes. Wood-rotting fungi variously secrete one or more of the four extracellular ligninolytic enzymes (laccases and/or peroxidases) involved in the lignin degradation in different combinations (Kinnunen *et al.* 2016). Moreover, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds must initiate changes to the lignin structure since the secreted enzymes exceed the micropore size of the intact cell wall initializing reactions only on exposed wood surfaces (Srebotnik and Hammel 2000, Dashtban *et al.* 2010, Yelle *et al.* 2014). Figure 5 summarizes the major enzymatic and non-enzymatic steps and the enzymes involved in lignin degradation by basidiomycetes white-rot fungi (Dashtban *et al.* 2010). The enzymatic system is more closely presented in 2.3.2.



Lac: laccase, LMS: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and $^{\bullet}OH$: free hydroxyl radicals.

Figure 5. Schematic diagram of the enzymatic and non-enzymatic lignin transformations by basidiomycetes white-rot fungi: the major steps and enzymes involved. Reprinted with permission from Dashtban *et al.* (2010). Copyright (2010) e-Century Publishing Corporation.

Wood-decaying and litter-decomposing basidiomycetous fungi, along with some ascomycetes, are among the few microorganisms capable of fully degrading the complex lignocellulosic matrix (Hatakka 2001, Hatakka and Hammel 2010). Conventionally, they can be classified into three major groups according to the visually distinguishable type of decay they cause: white-rot and brown-rot mainly exerted by basidiomycetes, and soft-rot mainly caused by ascomycetes. White-rot fungi degrade lignin leaving decayed wood whitish in color and fibrous in texture, and the fungi can be further divided into two subtypes. Simultaneous degraders (e.g. *Trametes versicolor*, *Heterobasidion annosum* and *Irpex lacteus*) are the most common and they are involved in the oxidative cleavage of lignin and structural polysaccharides at similar rates. Selective delignifiers e.g. *Ceriporiopsis subvermispora*, *Phellinus* pini, *Phlebia* spp. and *Physisporinus rivulosus* (syn. *Obba rivulosa*) are less common, and they are capable of removing lignin in advance of cellulose and hemicelluloses (Hakala *et al.* 2004). By secreting extracellular oxidative enzymes to produce reactive oxygen species they degrade and modify lignin to heterogenous aromatics that are ultimately further metabolized by the fungi or other organisms. White-rot fungi account for more than 90% of all wood-rotting basidiomycetes (Hatakka and Hammel 2010). White-rot fungi are more commonly found on angiosperm (hardwood) than on coniferous gymnosperm wood species in nature. Coniferous guaiacyl lignin is typically more resistant to microbial decomposition than syringyl lignin (Cornwell *et al.* 2009).

Brown-rot fungi account for less than 10% of the taxonomic diversity of lignocellulose degrading basidiomycetes but are widespread in nature. Especially in northern coniferous forest ecosystems mostly consisting of gymnosperms, they represent the dominant wood decay fungi (e.g., *Gloeophyllum trabeum, Postia placenta, Piptoporus betulinus*). Brown-rot fungi are thought to have lost their ligninolytic and cellulolytic enzyme system during evolution (Floudas *et al.* 2012, Cragg *et al.* 2015). They initiate cellulose depolymerization nonenzymatically, using highly reactive hydroxyl radicals (HO•) generated by Fenton chemistry (1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^- \qquad (1)$$

The fungal hyphae produce hydrogen peroxide (H_2O_2), oxalic acid and iron-reducing compounds such as methoxylated quinones. The oxalic acid stabilizes Fe^{3+} as a complex which diffuses into cell wall along with H_2O_2 and the reducing compounds, producing a highly reactive oxidative system. In this process lignin is modified by hydroxylation and demethylation of the aromatic rings with increased content of alpha-carbonyl functionalities and extensive repolymerization, but it is not metabolized by the fungi and instead persists in the soil (Hatakka and Hammel 2010, Cragg *et al.* 2015).

Soft-rot fungi (e.g. *Daldinia concentrica, Aspergillus niger* and *Penicillium chrysogenum*) typically degrade wood in wet environments and cause characteristic decay patterns for example by forming chains of diamond-shaped cavities that generally follow the orientation of the secondary cell wall cellulose fibrils or by eroding the secondary wall completely leaving a relatively intact middle lamella. They show preference for cellulose and hemicellulose with moderate alterations of lignin (Hatakka 2001, Hamed 2013).

2.3.2 OXIDOREDUCTASES IN LIGNIN CONVERSION

White-rot fungi are the most effective for delignification due to the production of ligninolytic extracellular oxidative enzymes, namely oxidoreductases, and they have potential in several industrial and biotechnological processes as they are highly non-specific and thus capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. Lignin-degrading fungi produce three extracellular heme peroxidases: lignin peroxidase (LiP, EC 1.11.1.14), manganese-dependent peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16). These belong to class II secreted fungal peroxidases, which are members of the superfamily of plant and microbial peroxidases. Recently found superfamilies of heme peroxidases are dye-decolorizing peroxidases (DyPs, EC 1.11.1.19) (Hofrichter *et al.* 2010, Liers *et al.* 2011) and heme-thiolate peroxidases (HTP, EC 1.11.2-) including unspecific peroxygenases (UPOs, EC 1.11.2.1) which catalyze the transfer of peroxide-oxygen to substrate molecules (Kinne *et al.* 2011). Another major group of enzymes involved in lignin degradation are the copper-containing phenol oxidases, laccases (EC 1.10.3.2 benzenediol:oxygen oxidoreductase) as well as several other oxidoreductases that function as auxiliary supporting enzymes (unable to degrade lignin on their own) involved in, for example, hydrogen peroxide production for peroxidases or decreasing the amount of radicals formed in delignification. These include glyoxal oxidase (GLOX, EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (GOX, glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (QR, EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18).

In addition to oxidative enzymes, hydrolytic enzymes such as feruloyl esterases (EC 3.1.1.73) are involved in the release of plant aromatic compounds. In addition to secreted enzymes, fungi also produce intracellular enzymes, such as cytochrome P450 monooxygenases (CYPs, EC1.14.14.1), that take part in the ligninolytic process by metabolizing the potential toxic aromatics derived from the degradation process to less toxic metabolites (Mäkelä *et al.* 2015). A newly found class of fungal ligninolytic enzymes is glutathione dependent β-transferases (GST-etherases, GSTs; EC 2.5.1.18), that catalyze the reductive cleavage of the α–oxidized β–aryl ether linkage (Morel *et al.* 2009). Also non-enzymatic mechanisms participate in the conversion of lignocellulose, which can be achieved with the aid of reactive oxygen species generated via Fenton reactions.

The delignification system and the role of enzymes involved in this process is complex and not completely understood and numerous review articles have been published on fungal degradation of lignocellulose and lignin (Leonowicz *et al.* 1999, Hatakka 2001, Higuchi 2004, Wong 2009, Hatakka and Hammel 2010, Cragg *et al.* 2015). White-rot fungi usually produce one or more ligninolytic enzymes in different combinations and they can be distributed into four groups according to their ability to easily produce laccases and peroxidases: 1) Laccase, MnP and LiP (*Trametes versicolor*, *Bjerkandera adusta*); 2) laccase and at least one of the peroxidases (*Lentinus edodes*, *Pleurotus eryngii*, *Ceriporiopsis subvermispora*); 3) only laccase (*Schizophyllum commune*); 4) only peroxidases (*Phanerochaete chrysosporium*) (Coelho-Moreira et al. 2013). Sequencing of fungal genomes has revealed that all white-rot fungi analyzed so far possess genes encoding ligninolytic peroxidases while genes encoding laccases are not always present (Floudas *et al.* 2012, Levasseur *et al.* 2014). In the cultivation media of white-rot fungi species the most

frequently observed lignin modifying enzymes are laccases and MnP, while LiP and VP are less common (Kinnunen *et al.* 2016).

2.3.3 CARBOHYDRATE DEGRADING HYDROLASES AND OXYGENASES

Three types of hydrolyzing enzymes (glycoside hydrolases) act synergistically to break down cellulose to glucose by employing acid/base catalysis. First, endoglucanases (EC 3.2.1.4) hydrolyze random bonds in the cellulose chains, each cleavage event exposing a free reducing (C1) end and a non-reducing (C4) end. In the next step exoglucanases (EC 3.2.1.9; cellobiohydrolases) and other classes of cellobiohydrolases (EC 3.2.1.176) attack the exposed non-reducing ends moving along the chains and cleaving off cellobiose units. Finally, cellobiose is subsequently hydrolyzed to glucose by β -glucosidases (EC 3.2.1.21) (Pérez *et al.* 2002, Rytioja *et al.* 2014).
Hemicellulose backbone degradation is accomplished by the combination of endoglucanases, endoxylanases, and endomannanases, with side chain removal by xylosidases and arabinofuranosidases. The resulting oligosaccharides are further degraded by xylosidases, mannosidases, and galactosidases. Furthermore, feruloyl esterases are required to break the covalent linkages between hemicellulose and lignin, and acetylesterases to remove acetyl residues (Pérez *et al.* 2002, Rytioja *et al.* 2014).
It has been shown only recently that direct enzymatic oxidative processes also play a major role in the conversion of polysaccharides. This is carried out by so-called lytic polysaccharide monooxygenases (LPMOs) that enhance polysaccharide degradation by hydroxylating and breaking up glycosidic bonds. They attack directly on crystalline cellulose, xyloglucan, glucomannan, xylan, starch and chitin (Vaaje-Kolstad *et al.* 2010) and provide new chain ends as starting points for the hydrolases. LPMOs are copper-dependent mononuclear enzymes, and their activity depends on the presence of molecular oxygen and an electron donor (Quinlan *et al.* 2011). Electrons can be directly provided by extracellular cellobiose dehydrogenases (CDH) or small molecule electron donors (mediators), such as diphenols, polyphenols, hydroxycinnamic acids and 3-hydroxyanthranilic acid (3HAA), that are derived from plants and fungi. These mediators can be further regenerated by redox enzymes. Recently, it has been shown that insoluble high molecular weight lignin functions as a reservoir of electrons, facilitating the LPMO activity. The electrons are donated to the enzyme by a long-range electron transfer involving soluble low molecular weight lignins present in plant cell walls (Westereng *et al.* 2015, Kracher *et al.* 2016). However, so far there is no report on how lignin structure is modified during this process.

2.4 LACCASES

2.4.1 LACCASE PROPERTIES

Laccases are widely distributed in nature and they are found in fungi, plants, insects and some bacteria. They have different biological roles determined by their origin and the organism life stage. Plant laccases are involved in lignin biosynthesis and fungal laccases are involved in the opposite process, lignin biodegradation. Furthermore, laccases catalyze

processes in plant pathogenesis, pigment formation, oxidation of toxic compounds, wound healing etc. (Thurston 1994). Laccase was first described in 1883 by Yoshida, who found it in the sap of the Japanese lacquer tree, *Rhus vernicifera* (Yoshida 1883). Laccases were further characterized in 1985 as metal containing oxidases (Bertrand 1985). Oxidases are defined as enzymes, which catalyze oxidation reactions by using molecular oxygen as the electron acceptor and reducing it either to hydrogen peroxide or to water without the incorporation of oxygen atom(s) into the substrate. Laccases belong to a diverse superfamily of multicopper oxidases ('blue copper oxidases') that contain four copper atoms distributed in one mononuclear (T1) and one trinuclear (T2/T3) domain in the enzyme's active center. They are extracellular glycoproteins (glycosylation 10–50%) of about 500 amino acid in length (typically 60–110 kDa in monomers), occur as isoenzymes and may oligomerize to form dimers or multimeric complexes (Thurston 1994, Claus 2004, Baldrian 2006, Christopher *et al.* 2014). In Figure 6 the laccase structure from the white-rot fungus *T. versicolor* and the bacterial species *Streptomyces coelicolor* are visualized as 3D ribbon diagrams. High carbohydrate content is assumed to provide laccase thermostability, and also salt bridges and hydrogen bonding between the copper atoms directly affect the laccase stability (Hildén *et al.* 2009).

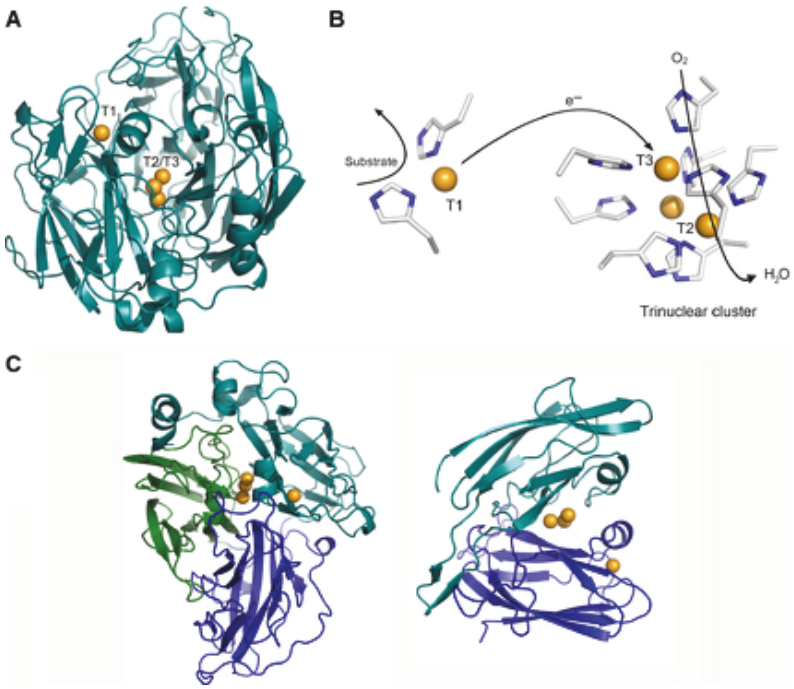


Figure 6 A. Ribbon diagram of laccase from *T. versicolor* showing two channels leading to the T2/T3 cluster; B. Laccase active site and; C. Comparison of two different size laccases *T. versicolor* and *S. coelicolor*. Reprinted with permission from Pollegioni *et al.* (2015). Copyright (2015) John Wiley and Sons, Inc.

The substrates are oxidized by one electron by the T1 copper domain and the extracted electrons are transferred intramolecularly, probably through a strongly conserved His-Cys-His tripeptide motif, to the trinuclear T2/T3 center (Ferraroni *et al.* 2007, Solomon *et al.*

2008). The stoichiometry is four molecules of reducing substrate for each molecular oxygen which is reduced altogether by four electrons (2).

$$4\ RH + O_2 \rightarrow 4\ R\bullet + 2\ H_2O \qquad (2)$$

First, substrates reduce the T1 site (Cu^{2+} to Cu^{+}) in the resting fully-oxidized enzyme, which then transfers the electrons one at a time to the Cu2/Cu3 trinuclear site resulting in a fully reduced state of the enzyme. Thereafter, a reduction of one oxygen molecule occurs at the trinuclear center through the formation of two oxygen-bound intermediates, peroxy- and oxy radical-intermediates, with two sequential electron transfers resulting in a formation of two molecules of water (Figure 7). The diffusion of dioxygen to the trinuclear site is rate limiting, followed by a rapid single electron transfer from T1. The slow decay of the peroxide intermediate is facilitated by the final electron transfer from the T2 copper, and is accelerated with decreasing pH and with protonation from a carboxylic acid residue near the active site. The second water molecule is released in the last step and all the copper atoms are fully oxidized (Lee *et al.* 2002, Wong 2009).

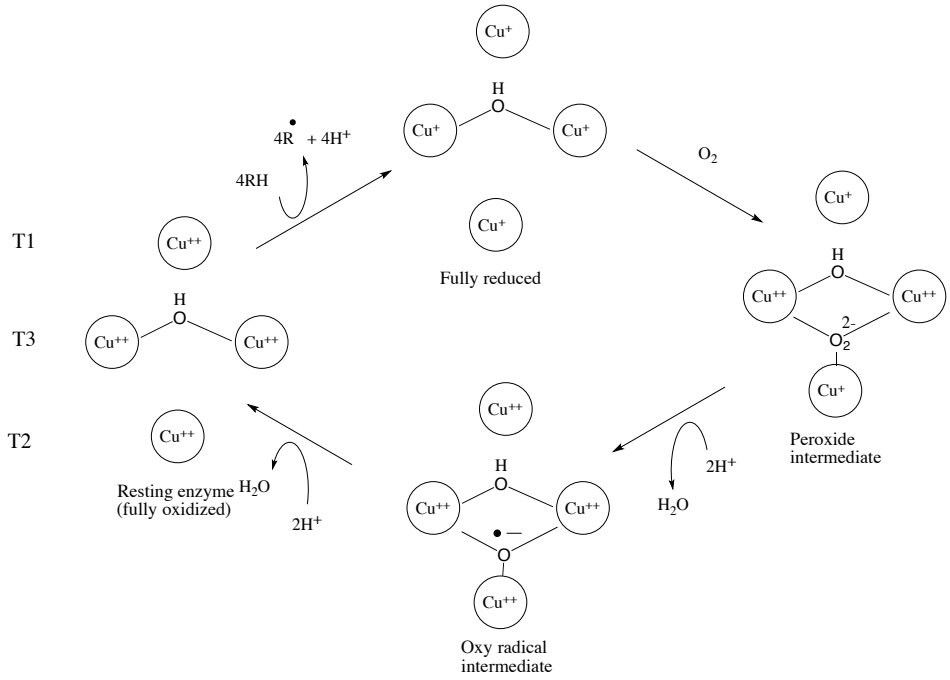


Figure 7. Schematic representation of catalytic cycle of laccase showing the mechanism of reduction and oxidation at the enzyme copper sites with four-electron reduction of a dioxygen molecule to water. Redrawn from Wong (2009).

Laccases possess moderately low redox potentials (E^o 0.4–0.8 V vs. NHE, Normal Hydrogen Electrode) compared to peroxidases, and from an electrochemical view they can be divided in low- and high-redox potential laccases (Mate and Alcalde 2015). The substrate binding site is located in a small, negatively-charged cavity close to the T1 site. The laccase redox potential is a result of a combination of various factors, such as T1 copper–ligand interactions, the effects of desolvation around the T1 site, the intermolecular electrostatic interactions, and the restrictions in protein folding (Li *et al.* 2004). The substrate oxidation

step involves the electron transfer from the reductant to the T1 copper site in the protein's active site. The catalytic efficiency of laccases has shown to be directly proportional to the difference of redox potential of the T1 copper and the substrate donor (Xu 1996, Xu *et al.* 2000, Pogni *et al.* 2015) i.e. the higher the potential difference between the T1 site acceptor and the substrate donor, the higher is the catalytic efficiency (k_{cat}/K_m). Laccase activity is dependent on pH and fungal laccases typically exhibit pH optima in the acidic pH range (pH, 3-6), and plant and bacterial laccases function at neutral to alkaline range (pH 6-8). Under the catalysis of phenolic substrates, bell shaped pH profiles have been observed and their maxima were distinct for each laccase (Xu *et al.* 2000). Higher pH decreases the redox potential of phenols, but on the other hand, higher pH has been ascribed to hydroxide inhibition of the trinuclear cluster, decreasing the enzymatic activity (Xu 1997).

2.4.2 DIRECT OXIDATION BY LACCASE

Laccases can directly oxidize a wide range of electron-rich natural and industrially produced organic substrates, such as phenols and polyphenols, aromatic amines, thiols and heterocyclic compounds (Xu 1996) to the corresponding radicals and even inorganic and organometallic compounds such as iodide, Mn^{2+} chelates, $[W(CN)_8]^{4-}$ and $[Fe(EDTA)]^{2-}$ (Baldrian 2006, Morozova *et al.* 2007). The oxidative ability of fungal laccases on lignin is restricted by the size and oxidation potential of reacting substrates (Tadesse *et al.* 2008). The laccases have been reported to oxidize substrates with redox properties somewhat higher (approximately 0.2-0.3 V) than those of laccase itself (Bourbonnais *et al.* 1998, Tadesse *et al.* 2008). The laccase reactivity decreases with the increase of the substrate size (d'Acunzo *et al.* 2002). Substrate solubility has a significant effect on the reactivity, and many water soluble organic co-solvents can be used to increase the substrate solubilities, such as methanol, ethanol, 1,4-dioxane, acetone, etc. and laccases usually tolerate moderate or even high solvent concentrations quite well (d'Acunzo *et al.* 2004). Laccase enzymes must be in direct contact with their substrate molecules in order to carry out oxidation but on the other hand, the close interaction with cell wall components is restricted, as illustrated in Figure 8.

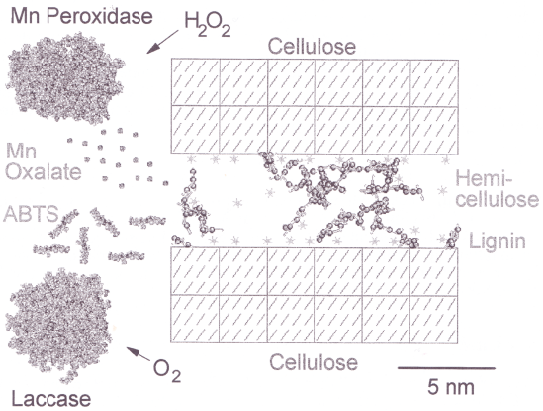


Figure 8. Comparison of the sizes of some components in various putative lignin biodegradation systems with the pores in the secondary wall model. Reprinted with permission from Jurasek (1998). Copyright (1998) American Chemical Society.

In general, laccases are able to effectively and directly oxidize only the small phenolic lignin fragments or exposed lignin phenolic subunits that can be accessible to the laccase active site. Thus they are capable of the initial oxidation or cleavage of phenolic lignin units that often comprise around 10 - 20% of the polymer (Adler and Hernestam 1955, Martínez *et al.* 2005). Non-phenolic parts of lignin possess redox potentials of above 1.3 V that are usually above laccase's oxidation capacity and cannot be directly oxidized by laccase.

Oxygen centered reactive radical intermediates can be generated by the direct oxidation of phenolic units. These radicals may further undergo various spontaneous follow-up reactions independent of enzyme activity, such as self-coupling reactions leading to the aromatic coupling with formation of new C-C and C-O-bonds with increased polymerization effect (Areskogh, Li, Gellerstedt, *et al.* 2010, Areskogh, Li, Nousiainen, *et al.* 2010, Areskogh, Nousiainen, *et al.* 2010, Maijala *et al.* 2012). The degradation of the phenolic end-groups may occur through aryl-alkyl cleavage with formation of the methoxylated hydroquinones that can be further oxidized to the corresponding quinones. Also, cleavage of the Cα-Cβ-bonds with the formation of corresponding carboxylated aromatics has been suggested as well as Cα-oxidation products. Figure 9 illustrates the lignin phenolic end-group transformations proposed for laccase-catalyzed reactions (Crestini and Argyropoulos 2001, Higuchi 2004). All intermediates may react further in laccase-catalyzed or other types of transformations. The ability of laccase to contribute to lignin degradation has been challenged for decades, as laccases have no direct ability to break up lignin. On the other hand, in nature fungi are found e.g. *Pycnoporus cinnabarinus*, which apparently rely predominantly on laccases in lignin degradation (Eggert *et al.* 1997). However, recent studies have shown that *P. cinnabarinus* has gene-encoding ligninolytic peroxidases (Levasseur *et al.* 2014).

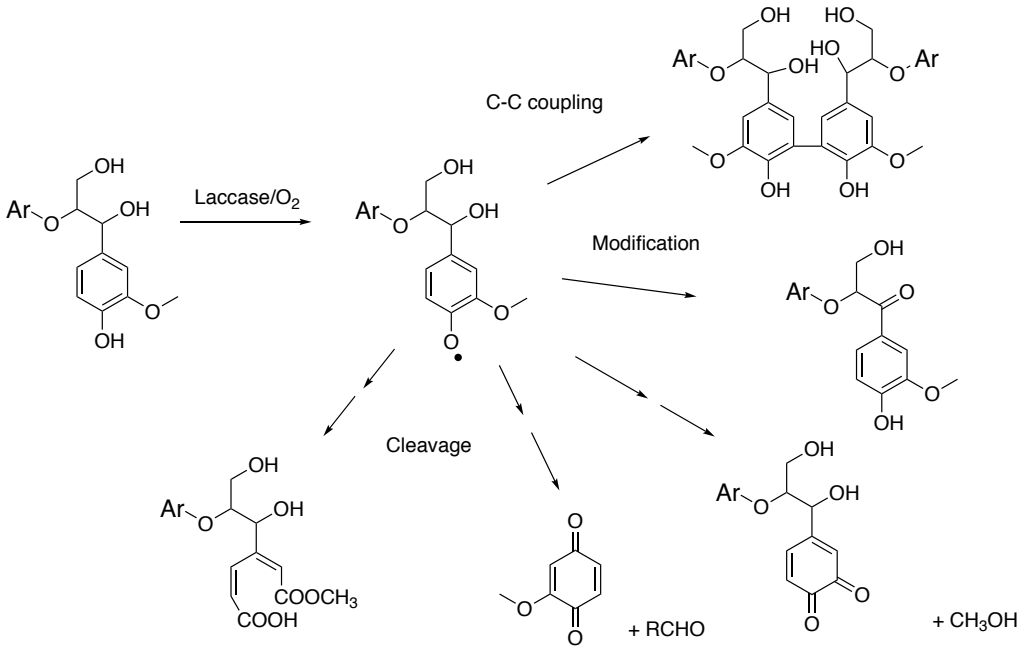


Figure 9. Generally proposed pathway for the laccase oxidation of lignin phenolic units.

The research on laccases has revealed that some low molecular weight compounds that can be oxidized by laccase to stable radicals, may act as redox mediators. In the follow-up reactions they can act as electron shuttles to transfer the oxidation to more recalcitrant substrates, e.g. non-phenolic lignin moieties or environmental xenobiotics that in principle are not substrates for laccase, because of their incompatible redox potential (Bourbonnais and Paice 1992, Bourbonnais *et al.* 1997, Morozova *et al.* 2007). The biotechnological applicability of laccase may therefore be extended using a laccase-mediator system (LMS) as originally studied by, for example, Call and Mücke (1997).

2.4.3 INDIRECT OXIDATION BY A LACCASE –MEDIATOR SYSTEM

Redox mediators (co-catalysts or enhancers) have been defined as small molecules that perform as shuttles between the oxidant and the target substrate. In laccase-mediator – system the mediator is continuously oxidized by laccase, with molecular oxygen as the oxidant, and is subsequently reduced by the substrate. The catalytic cycle of a laccase-mediator system (LMS) is represented in Figure 10.

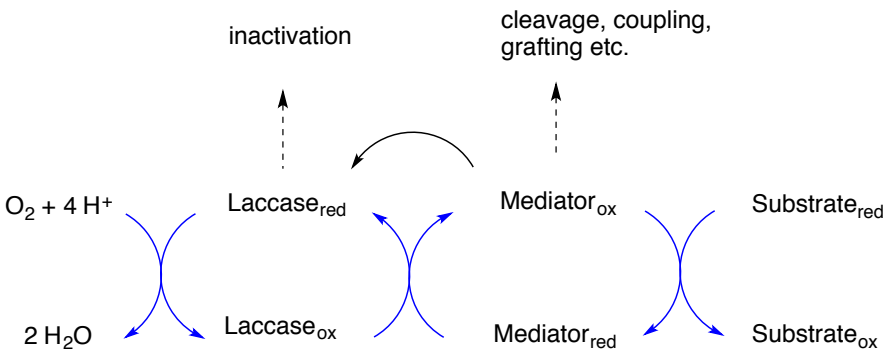


Figure 10. Schematic representation for laccase-catalyzed oxidation of substrate in the presence of a mediator and the potential undesired side-reactions of mediators, adapted from Roth and Spiess (2015).

The ideal mediator should be a good substrate for laccase, it should be stable in its oxidized form and perform as many redox cycles as possible without degrading or reacting by itself producing unwanted side reactions. It should possess high oxidation capacities but it should not inhibit or degrade the enzyme. Also, the mediators should be inexpensive to enable their use as industrial co-catalyst and they should not be (or produce) toxic components from an environmental point of view.

The molecules capable of transferring the oxidation between oxidized laccase and secondary substrate may be organic compounds or inorganic salts. So far over 100 compounds have been studied or patented as mediators, but most of them have proved inefficient due to the instability of the mediator intermediates, which results in incomplete redox cycles and poor substrate oxidation (Bourbonnais *et al.* 1998, Díaz González *et al.* 2009, González Arzola *et al.* 2009). Examples of some synthetic compounds that have been studied as mediators are presented in Figure 11.

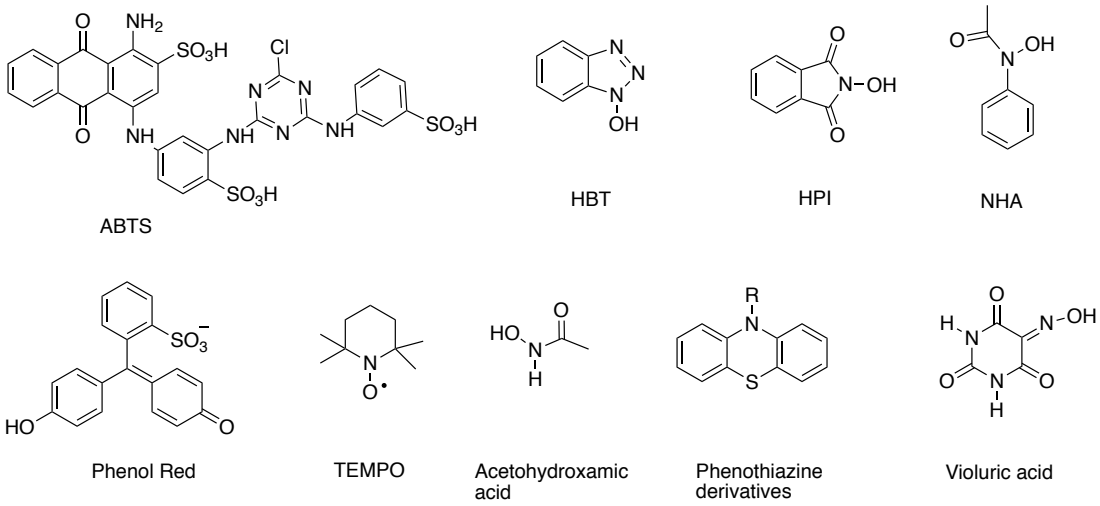


Figure 11. Some synthetic compounds suggested as mediators. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)]; HBT (1-hydroxybenzotriazole); HPI (N-hydroxyphtalimide); NHA (N-hydroxyacetamide); TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical).

The laccase mediator system (LMS) was first described by Bourbonnais and Paice (1990) with the use of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] as a mediator in the oxidation and cleavage of non-phenolic β-1 and β-O-4 lignin model dimers by laccase (Bourbonnais 1990). The delignification of unbleached Kraft pulp by laccase can be supported by several low-molecular mass dyes or aromatic (hydrogen) donors, and up to 50% delignification can be reached by a laccase–mediator system (Bourbonnais *et al.* 1995, 1997). Several laccases are able to oxidize ABTS first to the stable cation radical $[ABTS]^{+\bullet}$, forming an intense blue-green color that has often been used to measure enzyme activity, and then the radical cation will be slowly further oxidized to dication $[ABTS]^{2+}$. The redox potentials of $[ABTS]^{+\bullet}$ and $[ABTS]^{2+}$ were estimated as 0.68 V and 1.09 V (vs. NHE), respectively (Scott *et al.* 1993). The latter has been described as the actual mediator for oxidation of more recalcitrant lignin substrates, whereas the cation radical reacts only with phenolic substructures in lignin (Bourbonnais *et al.* 1998).

The mediator–substrate oxidation has been reported to occur via three mechanisms that are presented in Figure 12. The ABTS mediator–substrate oxidation has been suggested to proceed via an electron transfer mechanism (ET) (Figure 12 A). The Cα-carbonylation process competes with Cα-Cβ-cleavage during the oxidation of non-phenolic substrates in the LMS. The Cα-carbonyl products are preferentially produced in reactions involving the HAT mechanism shown in Figure 12 B (e.g. HBT, phenolic mediators) and the ionic mechanism shown in Figure 12 C (e.g. TEMPO and its analogs), whereas oxidations involving ET mechanism (e.g. ABTS) give the Cα-carbonyl product as well as the Cα-Cβ-cleavage products (Baiocco *et al.* 2003). The ET mechanism requires substrates with suitable oxidation potential, and the HAT mechanism requires substrates with relatively weak C-H bond.

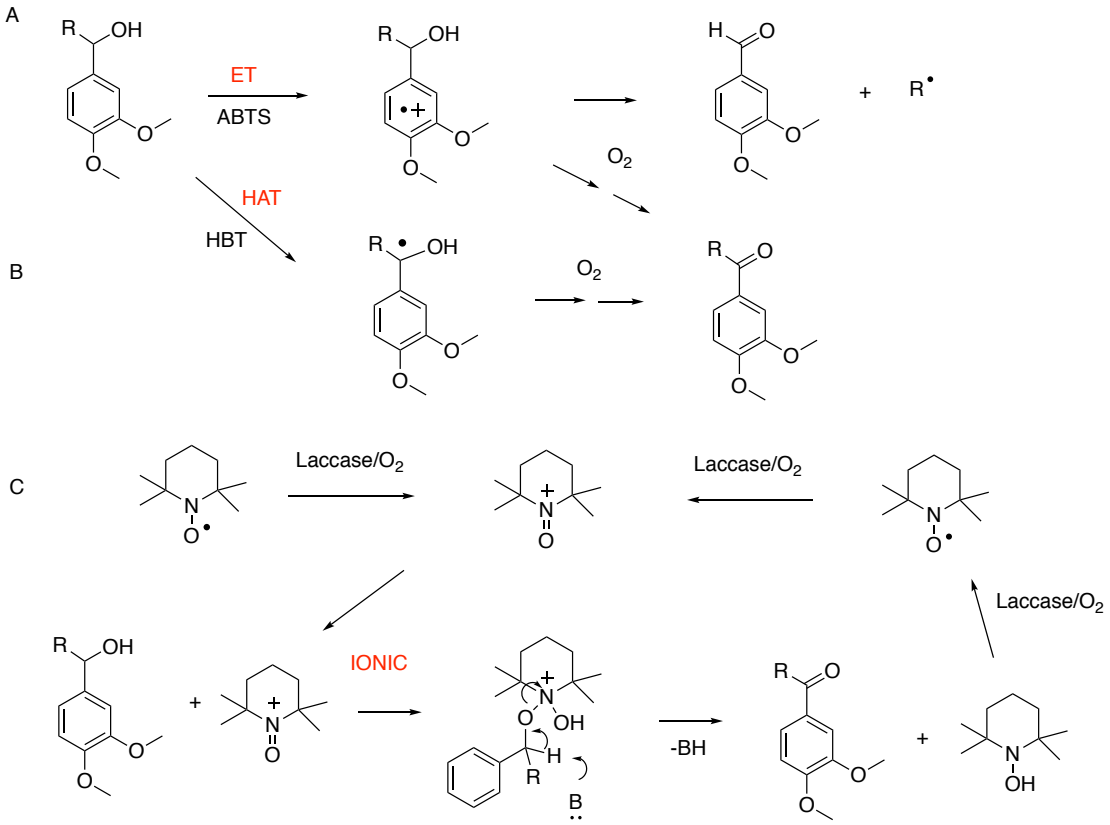


Figure 12. Proposed mechanisms for laccase-catalyzed mediated oxidation: A) Electron transfer (ET); B) Hydrogen atom transfer (HAT); C) Ionic mechanism

Most of the mediators have been synthetic compounds based on nitrogen heterocycles such as TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical) or HBT (1-hydroxybenzotriazole). HBT has been used most commonly as reagent in peptide synthesis, but is also known as one the most effective mediators in pulp bleaching. Kawai *et al.* (2002, 2004) reported in their early studies with HBT and laccase from *Trametes versicolor* that the oxidation of a non-phenolic β-O-4 lignin model dimer, 1-(4-ethoxy-3-methoxyphenyl)-1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)propane produces products with Cα-Cβ cleavage, Cα-oxidation, β-ether cleavage and aromatic ring cleavage. These diverse cleavage products are usually reported for LiP -type oxidation with an initial electron transfer from the aromatic ring, and the formation of radical carbocation. Nowadays it has been confirmed, that HBT performs the oxidation preferentially using HAT (hydrogen atom transfer) and proceeds by abstracting hydrogen from the prevailing benzylic positions of lignin (Fabbrini *et al.* 2002, Cantarella *et al.* 2003). Examples of similar N-OH -type mediators are N-hydroxyacetamide (NHA), N-hydroxyphtalimide (HPI), acetohydroxamic acid and violuric acid (VLA) (See Figure 11). The common organic oxidant, TEMPO, and its analogues react faster with primary alcohols than with secondary alcohols, which is in line with the proposed ionic mechanism. TEMPO is also capable of oxidizing primary alcohol moieties in carbohydrates (Arends *et al.* 2006).

So far, the exploitation of laccase-mediator systems (LMS) in industrial processes has been limited, since they are expensive in the amounts required in oxidation processes and they are mostly possible threats to the environment as chemicals. Recently, it has been found that

some natural substances, especially derived from syringyl -type plant phenolics, are good substrates for laccases with long enough life-time to mediate the oxidation to substances which are recalcitrant towards oxidation by laccases (Cho *et al.* 2004, Camarero *et al.* 2005, Camarero *et al.* 2007). These phenolic compounds may be produced from oxidized lignin units by fungi themselves (e.g. syringaldehyde, vanillin and substituted hydroquinones), plant phenolics present in plants (sinapic, ferulic and p-coumaryl acid) as lignin carbohydrate complexes, plant secondary metabolites or secondary, extracellular fungal metabolites (e.g. 3-hydroxy-anthranilic acid (3-HAA) and 4-hydroxybenzoic acid). It is also possible that fungi in themselves are able to produce phenolic compounds *de novo* (Kirk and Farrell 1987, Rogalski and Niemenmaa 1996). For example, the white-rot fungus *P. cinnabarinus* produces 3-HAA as fungal metabolite, which is considered to be an effective natural laccase mediator (Eggert *et al.* 1996). The simple syringyl-type phenolics are potentially cheap and readily available from, for example, downstreams of industrial hardwood pulping wastes (Camarero *et al.* 2007, Canas and Camarero 2010). Being biodegradable, natural phenolic compounds potentially also react further in laccase catalyzed reactions, and thereby result in environmentally more sustainable processes. Examples of phenols of natural origin and some of their simple derivatives are presented in Figure 13.

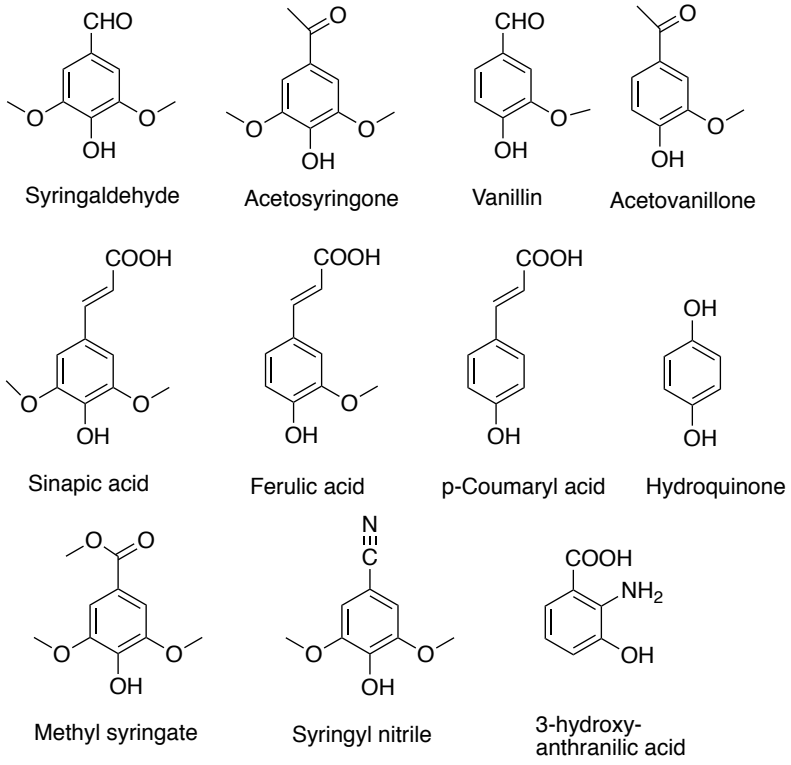


Figure 13. Some natural mediators and their derivatives

One of the first natural laccase mediators discovered was syringaldehyde. Kawai and Umezawa (1989) showed that syringaldehyde was able to act as a laccase mediator oxidizing monomeric non-phenolic lignin model compounds, veratryl alcohol and 3,4,5-trimethoxybenzyl alcohol to the corresponding aldehydes in some extent. The degradation products of syringaldehyde were identified as the corresponding hydroquinone and

benzoquinone (Kawai and Umezawa 1989). In their study (2005) Camarero *et al*. screened the effectiveness of 44 compounds of natural origin using recalcitrant dye Reactive Black 5 as substrate and found acetosyringone and syringaldehyde in particular but also *p*-coumaric acid, vanillin, acetovanillone and methyl vanillate, to be the most effective mediators in oxidizing recalcitrant dyes (Camarero et al. 2005).

As natural substrates for laccases, the phenolic compounds are supposed to be readily oxidized, but in order to form stable radicals, both *ortho* and *para* positions should be blocked by groups which give increased steric protection or resonance stabilization. In substituted syringyl-type phenols such as syringaldehyde or acetosyringone, both of the phenolic ortho-positions are occupied (as compared to corresponding guaiacyl or p-coumaryl -type phenols) thereby blocking the primary 5-5'- coupling reaction of the phenoxy radicals. The syringyl-type compounds possess two electron-donating methoxy substituents *ortho* to the phenolic position. The electron-donor substituents (e.g. OMe, OH) lower the redox potential of the phenols (compared to non-substituted ones) thereby making the enzymatic oxidation easier, while the presence of electron-withdrawing functionalities (e.g. COR) has the opposite effect (Bordwell and Cheng 1991, Tadesse *et al.* 2008, González Arzola *et al.* 2009). Both electron donors and acceptors conjugatively stabilize the phenoxy radicals. The stability of phenoxy radicals is also increased by steric hindrance of the *ortho* substituents to the phenol OH group (Morozova *et al.* 2007, Martorana *et al.* 2013).

The phenol oxidation rate is influenced especially by the pKa of the phenol and pH of the reaction medium, since the one-electron oxidation of phenoxide is thermodynamically preferred over oxidation of the corresponding phenol and even a slight degree of ionization can contribute significantly to its rate of reaction (Camarero *et al.* 2005, Litwinienko and Ingold 2007). For example, the measured oxidation potentials of, for example, syringaldehyde and acetovanillone are at pH 6 significantly lower than at pH 4, (542 mV and 534 mV vs. 720 mV and 693 mV, respectively) (Díaz González *et al.* 2009, González Arzola *et al.* 2009). The redox potential of the active site does not change with pH, but the enzyme structure surrounding the T1 copper atom is affected by the pH. It has been proposed, that the oxidation of phenols by laccases is dependent on a negatively charged residue close to the substrate-binding site of the enzymes that has a role in facilitating the formation of the phenoxy radical and allowing its stabilization during the catalytic reaction (Kallio *et al.* 2009). All fungal laccases have been reported to possess a conserved aspartic or glutamic acid residue close to the active site, e.g. an aspartate residue in fungal *Trametes versicolor* laccase (Piontek 2002) or glutamate in *Melanocarpus albomyces* laccase (Kallio *et al.* 2009). Rosado *et al.* (2012) compared the efficiency of bacterial and fungal laccases with syringyl-type mediators and showed that due the absence of this residue in bacterial laccases e.g in CotA laccase from *Bacillus subtilis*, the maximal reaction rates occur at alkaline pH values, where the concentration of the phenol deprotonated form is higher (Rosado *et al.* 2012). The action of phenols as laccase mediators was shown to be independent of the laccase used, but the reactivity vs. stability of the phenoxy radical formed has a significant effect on the on the efficiency of the mediated reaction.

Electrochemical techniques have been applied to study laccase-mediator systems to evaluate their redox process, e.g. the redox potentials of the reacting species, the ability to oxidize the substrates and the stability of the radicals generated. Diaz Gonzalez *et al*. (2009) studied the electrochemical behavior and the catalytic efficiency of sixteen phenolic mediators and their potential as mediators at pH 4 using cyclic voltammetry. They found that the most of the

compounds described as laccase mediators are not strictly redox mediators, since their oxidized intermediates are electrochemically unstable (not regenerated via direct electron transfer) with subsequent unproductive side reactions like degradation or polymerization. However, when the oxidations were performed in the presence of veratryl alcohol as non-phenolic lignin model, the radical generated at the electrode surface reacts rapidly to oxidize veratryl alcohol, and the mediators were regenerated. Thus it was concluded that all the sixteen phenolic compounds studied have a catalytic effect on veratryl alcohol oxidation (Díaz González *et al.* 2009). According to a cyclic voltammetric study by Gonzalez-Arzola *et al.* (2009) with eighteen natural and synthetic compounds in an aqueous buffered medium at pH 6, the behavior of phenols differed considerably, because in these reaction conditions practically no polymerization on the electrode surface was observed (González Arzola *et al.* 2009). Furthermore, at pH 6 the results suggested that acetovanillone, a natural mediator, is even more effective than the synthetic mediators TEMPO, violuric acid or ABTS in oxidizing veratryl alcohol and Kraft lignin. Also, it was concluded, that the redox potentials of the phenolic mediators seem to have a negligible relevance on their catalytic efficiency and this further supported the general HAT oxidation mechanism, proposed for action of phenolic mediators (Díaz González *et al.* 2009). The ability to form relatively stable oxidized intermediates after their first oxidation step together with steric issues determines the efficiency of these mediators/enhancers.

The stability of the phenoxy radical has been shown to have a major importance on mediated oxidation reactions, and furthermore, it has been suggested the stability of phenoxy radicals is improved by the presence of electron donor groups at the para-position, while electron-withdrawing substituents lead to a decreased radical stability (Díaz González *et al.* 2009, Rosado *et al.* 2012). In Rosado *et al.* (2012) the oxidation intermediates and products of three phenolic mediators, syringaldehyde, acetosyringone and methyl syringate, were studied by using chromatographic and spectroscopic methods. With all three mediators, and especially syringaldehyde and acetosyringone, the compound 2,6-dimethoxy-p-benzoquinone was identified as one final oxidation product. The oxidative side reactions of phenols include phenol coupling reactions forming C-C and C-O dimers that can be further oxidized and therefore product mixtures are usually formed. Thus, different types of C-O radical coupling products were identified with several dimeric and trimeric structures. The findings suggested the typical formation of dimers through phenoxyl radical coupling on aromatic C1- or C3, with release of the carbonyl side group or methanol, respectively, or via phenoxy-radical addition on (hydro)quinone C2 (Rosado *et al.* 2012).

2.5 LIGNOLYTIC PEROXIDASES

Peroxidases appear throughout the biosphere and catalyze the oxidation of various substrates at the expense of peroxide as an electron acceptor and they produce water as a by-product. Peroxidases are found in plants, animals and microorganisms. They are involved for example in lignin degradation and lignin biosynthesis, in a plant growth hormone metabolism, in prostaglandin biosynthesis, and in the production of thyroxine and neutrophil-mediated detoxification reaction (Conesa *et al.* 2002, Martinez 2002, Hofrichter *et al.* 2010). They all are single polypeptide chains in the range of 30 to 40 kDa and contain a single ferric protoporphyrin IX as the prosthetic group. Based on a sequence analysis and X-ray crystallographic structures, the non-mammalian peroxidases have been grouped into

three classes: intercellular (Class I), extracellular fungal (Class II), and extracellular plant peroxidases (Class III). The extracellular enzymes are glycosylated and they contain two calcium-binding sites and at least four disulfide bonds presumably for the additional stability required for the secreted enzymes. The 3D structures of manganese and versatile peroxidases from *P. chrysosphorium* and *P. eryngii*, respectively are presented in Figure 14 A and B (Pollegioni *et al.* 2015).

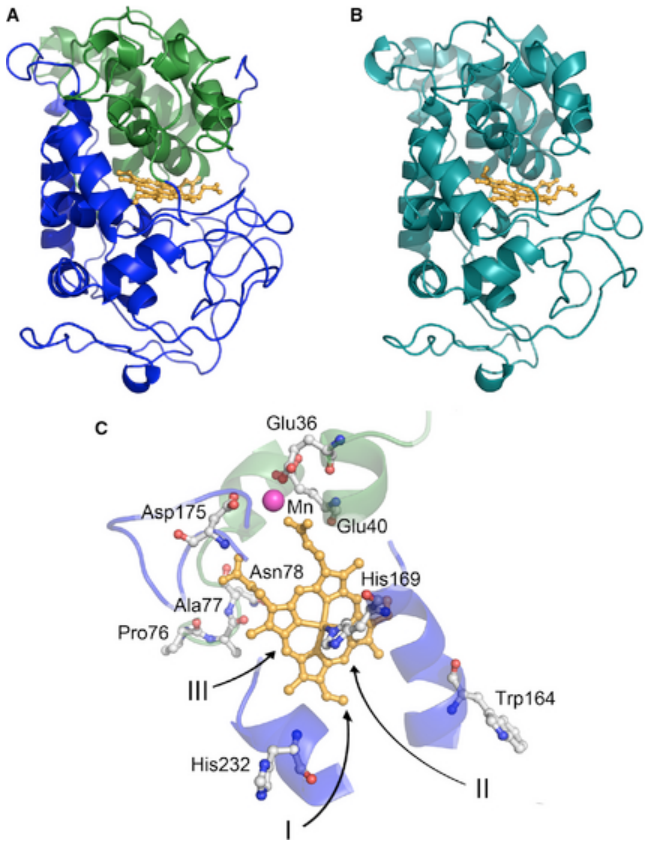


Figure 14. Structure of A) manganese and B) versatile peroxidases from *P. chrysosphorium* and *P. eryngii*; C) shows the axial view of the heme region of VP from *P. eryngii* and the putative long range electron transfer (LRET) pathways I, II and III. Reprinted with permission form Pollegioni *et al.* (2015). Copyright (2015) John Wiley and Sons, Inc.

Ligninolytic extracellular peroxidases have the advantage of having higher redox potentials and thus also a broader substrate range than laccases. Ligninolytic peroxidases contain a heme prostethic group (iron(III) protoporphyrin IX) in their active center. Their catalytic cycle involves the two-electron oxidation of iron(III) porphyrin IX by hydrogen peroxide (H_2O_2) to give the highly reactive oxoiron(IV) porphyrin IX π-cation radical (compound-I), which returns to its resting state after two consecutive one-electron reductions via compound-II -intermediate by the electron donating substrates (Ruiz-Dueñas *et al.* 1999). The catalytic cycle of MnP is presented in Figure 15. Ligninolytic extracellular peroxidases perform catalytic reactions using H_2O_2 as the electron acceptor with concomitant release of water, and they function via one-electron transfer oxidations of their substrates which can be small phenolic compounds, transition metals, etc. (Camarero *et al.*, 1999; Ertan *et al*., 2012; Ruiz-Dueñas *et al*., 1999). Although the heme active sites of these enzymes are structurally very similar and all contain a heme prostethic group (iron(III) protoporphyrin IX) at their

active sites to bind hydrogen peroxide as well as less reactive organic hydroperoxides, their reaction mechanisms are significantly different.

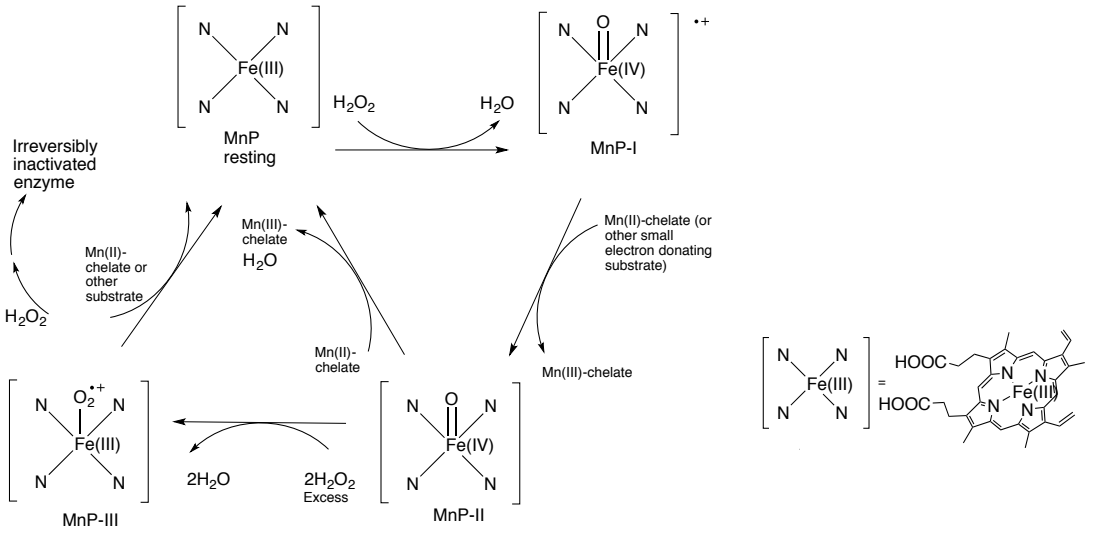


Figure 15. Peroxidase catalytic cycle presented for MnP, modified from (Wong 2009). Excess of hydrogen peroxide leads to formation of compound-III intermediate which may either be regenerated by substrate or decay by an irreversible mechanism to inactive enzyme.

Most white-rot fungi secrete MnP and laccase while the production of LiP and VP is less common (Kirk and Farrell 1987, Hatakka and Hammel 2010). LiP-type enzymatic activity, catalyzed by LiPs and VPs, is responsible for the direct cleavage and degradation of lignin. VPs were first found from *Pleurotus* and *Bjerkandera* spp. (Martinez 2002) and are hybrids of LiP and MnP with a bifunctional characteristic (Ruiz-Dueñas *et al.* 2009). The LiP-type activity for VP (and LiP) on the other hand, has been shown to involve Trp171 at the surface of the enzyme via long-range electron transfer (LRET) to the heme of compound-I, possessing high-redox potential (1.2 V in pH 3) to oxidize the non-phenolic substrates, e.g. veratryl alcohol and also other high-redox potential substrates as Reactive Black 5 (Tsukihara *et al.* 2008). LiPs extract one electron directly from non-phenolic lignin moieties to form highly reactive aryl cation radicals, which subsequently undergo a variety of non-enzymatic reactions like cleavage of Cα-Cβ-bonds, ring cleavages and α-oxidations as well as re-polymerization reactions. Phenolic substrates have been found to be more readily oxidized than non-phenolic structures. The heme passageway of VP (and LiP) is too narrow for large molecules for direct interaction with the enzyme's active center, as shown by crystal structures of some LiP and VP (Martinez, 2002; Wong, 2009). Instead, the accessibility of small phenolic substrates in contact with LiP heme active center has been reported to inactivate the enzyme, especially when no higher redox potential substrates are readily available. VPs have been reported not to have such inactivation effect (Busse *et al.*, 2013; Ruiz-Dueñas *et al.*, 2009; Tinoco *et al.*, 2007), which makes these enzymes attractive for many applications for example in pulp and bleaching technologies. Veratryl alcohol has been considered working as mediating substrate for LiP transferring the oxidation further to lignin in close vicinity of enzyme. The life span of highly reactive radical cations is very

short, and the active radicals readily react with various organic molecules that they encounter.

Specifically, MnPs have a manganese-binding site in the vicinity of heme, where Mn^{2+} is oxidized to reactive Mn^{3+} (Figure 15). The stoichiometry is two molecules of reducing substrate for each H_2O_2 with a net two-electron reduction to water (3).

$$2\,Mn^{2+} + 2\,H^{+} + H_2O_2 \xrightleftharpoons{MnP} 2\,Mn^{3+} + 2\,H_2O \qquad (3)$$

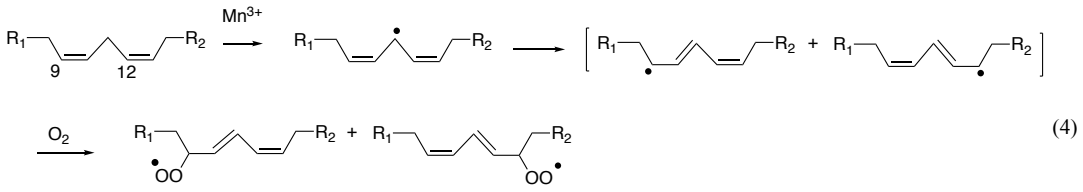
In the case of MnP-activity, Mn^{3+} requires organic diacids like oxalic, malonic or lactic acid to stabilize the highly reactive Mn^{3+} by chelation. The chelated Mn^{3+} serves then as a freely diffusible oxidant, a mediator, in solution at locations remote from the enzyme's active site. The complex formation stabilizes Mn^{3+} and, consequently the complexes have significantly lower oxidation capacities when compared to non-chelated Mn^{3+}. The bidentate ligated Mn^{3+} usually have redox potentials of around 0.7–0.9 V (Cui and Dolphin 1990, Armstrong 2008). The degradation of recalcitrant non-phenolic compounds has been limited with MnP generated Mn^{3+}chelates, due to their lower oxidation power. Thus MnPs are able to oxidize only the phenolic substructures in lignin with formation of phenoxy radicals to react further through non-enzymatic transformations like bond cleavages, α -oxidations, radical couplings and re-polymerizations (Wong 2009). Some mediators or co-oxidants, such as thiols, unsaturated fatty acids and their derivatives and some common laccase mediators e.g. 1-hydroxybenzotriazole (HBT), have been reported to be effective in the oxidation reactions of more recalcitrant compounds (Bermek *et al.* 2002, Hofrichter 2002, Michizoe *et al.* 2004, Kapich *et al.* 2005).

It has also been suggested that the chelating agents stimulate the rate of Mn^{3+} release from the enzyme (Kuan and Tien 1993). Wood-rotting fungi produce these organic acids as metabolites in significant amounts accompanied with the secretion of MnPs (Wariishi *et al.* 1992, Kishi *et al.* 1994). The Mn^{2+}/Mn^{3+} ion pair has a high redox potential in water (1.54 V) (Kishi *et al.* 1994), but the free nonchelated solvated Mn^{3+} ($Mn(H_2O)_6)^{3+}$) disproportionates quickly in water to Mn^{2+} and Mn^{4+}, and precipitates readily from solution as an insoluble oxide (MnO_2) being no more bioavailable for the enzyme. In practice, the MnO_2 accumulations have been found as black deposits in wood degraded by several white-rot fungi (Blanchette 1984).

Drawbacks of lignolytic peroxidases are their limited commercial availability and problematic activity. They require H_2O_2 but are also inhibited by an excess of peroxide (Taboada-Puig *et al.* 2011). The main limitation of all heme-peroxidases is their low operational stability, mostly due to their rapid deactivation by H_2O_2 with half-lives in the order of minutes in the presence of 1 mM H_2O_2. This means that the control of constant peroxide production is essential in order to carry out effective oxidation (Timofeevski *et al.* 1998, Bockle *et al.* 1999, Valderrama *et al.* 2002). Several approaches have been used in supplying the H_2O_2 into the reaction in different applications. Usually H_2O_2 has been added either in portions or gradually, but especially in larger scale this requires an effective distribution without producing high peroxide concentrations locally that inactivate the enzyme. The need for effective agitation may cause aggregation or denaturation of the proteins. Hydrogen peroxide has also been generated into the reaction *in situ* from β-D-glucose by flavoenzyme glucose oxidase (GOX, EC 1.1.3.4) producing D-glucono-δ-lactone

and H_2O_2, but this approach also causes problems due to substrate inhibition. In case of MnP also the need of manganese chelator and suitable co-oxidant restricts their use in biotechnical applications.

Peroxidation of unsaturated lipids has been proposed as the main depolymerization mechanism for MnP activity in white-rot fungi (Bao *et al.* 1994, Kapich *et al.* 2005, Fackler *et al.* 2007). According to Watanabe *et al*. (2000) the system involving MnP-dependent lipid peroxidation is an auto-oxidative self-propargating system where the peroxides are proposed to be produced according to Scheme 4. In the initiation step, the polyunsaturated lipid (RH) initially forms acyl radicals that stabilize to bis-allylic position by auto-oxidation to produce alkyl radicals (R•), which react very rapidly with any oxygen present to form peroxyl radicals (ROO•). In the MnP-dependent peroxidation of unsaturated fatty acid, lipid free radicals also produce superoxide radicals $O_2^{\bullet-}$ with subsequent disproportionation to generate H_2O_2.



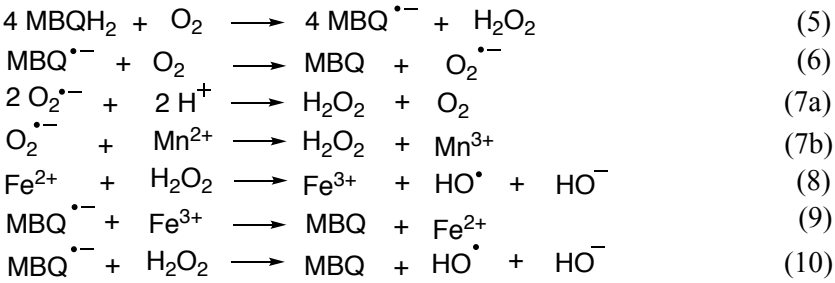
(4)

The actual oxidant in the MnP-lipid peroxidation system is not clear. Alkylperoxides decompose further in the presence of Mn^{3+} to produce alkoxyl radicals (RO•), which cleave into secondary oxidation products, finally producing a complex mixture of aldehydes, ketones, hydrocarbons, alcohols and cross-linked dimers (Watanabe *et al.* 2000). The alkoxyl radicals serve also as potential mediators in the reaction system. Kapich *et al.* (2005) suggested that lipid peroxidation oxidizes lignin β-O-4 structures by both hydrogen abstraction and electron transfer mechanisms, as they found also low amounts of LiP-type Cα-Cβ cleavage products in their reaction system. Ohashi *et al*. (2011) compared the reactivities of carbon-centred radical, peroxyl radical and alkoxyl radical towards non-phenolic monomeric and dimeric lignin model compounds. The observed reactivities were in the order alkoxyl > carbon >> peroxyl radical, and also the bond dissociation energy calculations for HAT supported these results. They suggested peroxyl radical as an important chain-carrying radical in lignin biodegradation with the actual reactive species being the alkoxyl and carbon radicals (Ohashi *et al.* 2011).

Laccases have also been reported to be involved in lipid peroxidation. A system comprising laccase and natural phenolic compounds, such as 4-hydroxybenzoic acid (HBA) or lignin, was found to rapidly and exhaustively peroxidize linoleic acid, particularly in the presence of manganese(II). These findings suggested that laccases may contribute to fungal lipid peroxidation *in vivo* thus expanding their role in the biodegradation of lignin and other recalcitrant aromatic compounds (Srebotnik and Boisson 2005).

Additionally, in the presence of iron, both peroxidases and laccases have found to participate on oxygen activation through the generation of the lignin-derived hydroquinones 2-methoxy-1,4-benzohydroquinone ($MBQH_2$) and 2,6-dimethoxy-1,4-benzohydroquinone ($DBQH_2$) and their further oxidation intermediates as semiradicals, which leads to the production of superoxide $O_2^{\bullet-}$, H_2O_2 and finally the generation of hydroxyl radicals HO• via

the Fenton reaction. In most biological systems iron is generally sequestered in redox-inactive complexes to prevent oxidative damage via the Fenton chemistry, but in the case of wood degradative conditions, enough iron and suitable chelators or reductants are present to solubilize the metal and enhance its bioavailability to facilitate the HO• radical formation. Gomez-Toribio *et al*. (2009) observed that the semi-quinone intermediates (as oxidation products of p-hydroxyphenyl, guaiacyl, and syringyl units of lignin) facilitate the redox-cycling of Fe(III) to Fe(II) in this system and serve as reducing agents for Fe(III) according to the following Schemes (5)-(10).



$$4\ MBQH_2 + O_2 \longrightarrow 4\ MBQ^{\bullet -} + H_2O_2 \quad (5)$$

$$MBQ^{\bullet -} + O_2 \longrightarrow MBQ + O_2^{\bullet -} \quad (6)$$

$$2\ O_2^{\bullet -} + 2\ H^{+} \longrightarrow H_2O_2 + O_2 \quad (7a)$$

$$O_2^{\bullet -} + Mn^{2+} \longrightarrow H_2O_2 + Mn^{3+} \quad (7b)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad (8)$$

$$MBQ^{\bullet -} + Fe^{3+} \longrightarrow MBQ + Fe^{2+} \quad (9)$$

$$MBQ^{\bullet -} + H_2O_2 \longrightarrow MBQ + HO^{\bullet} + HO^{-} \quad (10)$$

Marco-Urrea *et al*. (2010) showed the applicability of this fungal high oxidation power and low substrate specificity oxidizing system for the degradation of pharmaceutical recalcitrant organopollutants in wastewater.

2.6. ON THE HYDROGEN ABSTRACTION MECHANISM

Hydrogen atom transfer (HAT) reactions (11) are key steps in several important chemical and biological processes, from combustion and enzymatic catalysis to the chemistry of reactive oxygen species and antioxidants (Mayer 2011).

The oxidoreductase-catalyzed, mediated oxidations of benzylic lignin structures has shown to proceed via the HAT mechanism abstracting H-atom from the substrate benzylic α–position selectively to give a benzylic radical in the rate determining step (Baiocco *et al.* 2003). The benzylic radical is then transformed into oxidation products by interaction with the molecular oxygen present in the system (Crestini and Argyropoulos 2001). Lignin substrates, that have benzylic C–H bond energies (BDE) in the range of 85–90 kcal/mol, are easier to oxidize than those having non-benzyl aliphatic (BDE ca. 94–99 kcal/mol) or aromatic C–H bonds (BDE ca. 110–114 kcal/mol). In lignin β-O-4 structure the benzylic position with α-hydroxyl group has a C-H BDE of ca. 82–84 kcal/mol (Cantarella *et al.* 2003).

Hydrogen abstraction reactions are a specific case of atom transfer reactions where one electron and one proton are transferred in a single kinetic step from one group to another (11) (Warren and Mayer 2010).

$$X{-}H + Y\bullet \rightarrow X\bullet + H{-}Y \quad (11)$$

The rate constants of such reactions are related to their energetics, i.e. the difference between the bond dissociation energies for X-H and Y-H. Such energies do not involve strongly polar interactions and generally they have little solvent effect. Therefore, the reactions may be expected to have similar rate constants in the aqueous phase and in the

organic phase. Litwinienko and Ingold (2007) have shown that abstractions from RO-H bonds are very solvent dependent while abstractions from C-H bonds are not.
By definition, a hydrogen abstraction reaction is thermodynamically favorable, if the radical stabilization energy (RSE) value of the product radical is greater than that of the reactant radical. Moreover, where a species has several potential hydrogen abstraction sites, the most thermodynamically favorable site is that which results in the radical having the highest RSE. While kinetic factors can sometimes favor alternative reaction pathways, the consideration of RSE values can in the very least establish whether or not certain reactions are potentially feasible, and this in turn has important practical applications in chemistry and biology (Warren and Mayer 2010). Substituent effects can be interpreted as a combination of three different molecular orbital interactions: a) resonance stabilization with π-systems; b) hyperconjugation with adjacent C-H bonds; and c) stabilization through interaction with high lying orbitals describing lone pair electrons (Hioe and Zipse 2010). Simultaneous presence of donor and acceptor substituents at the same radical center implies an extra stabilization through "captodative" stabilization effects.
Carbon-centered radicals have a tendency to react with molecular oxygen present in the reaction system at diffusion controlled rate (10^9 $M^{-1}s^{-1}$) forming the peroxyl radical as intermediate species. It has been shown that benzylic radicals are reactive toward oxygen addition at rates of 10^{10} $M^{-1}s^{-1}$ (Crestini and Argyropoulos 2001). The radical may abstract second hydrogen from any available hydrogen donor (Figure 16) or decay by cleavage of superoxide (Crestini *et al.* 2003, Kapich *et al.* 2007). The superoxide radicals formed during this step would in turn react with phenoxy radicals, thus allowing the formation of further ring cleavage oxidation products (Crestini and Argyropoulos 2001, Reitberger *et al.* 2001).

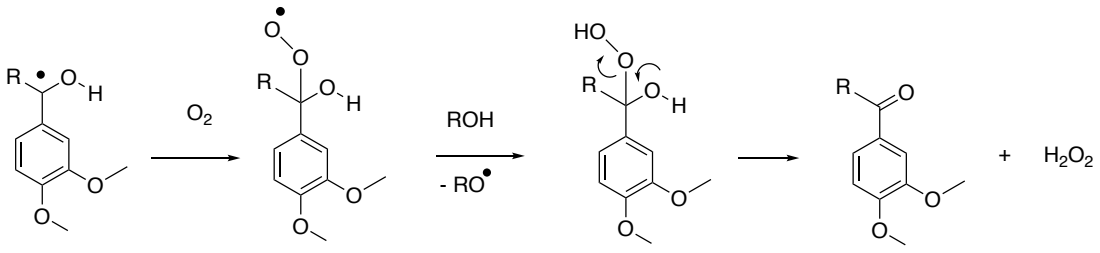


Figure 16. Suggested reaction path for benzylic radical reaction in presence of molecular oxygen with formation of corresponding α–carbonyl product and H_2O_2.

2.7. MOTIVATION FOR SELECTIVE α–OXIDATION IN LIGNIN AND LIGNIN MODELS

Catalytic oxidation processes, also including application of oxidative lignin modifying enzymes, are considered as the most promising techniques (besides gasification and thermochemical processes) for both functionalization and depolymerization of lignin in sulphur-free biorefining (Zakzeski *et al*. 2010, Lange *et al*. 2013). In this task especially the oxidative pretreatment methods have been most studied. Effective pretreatment techniques are needed for disentangling the cell wall matrix, as lignin has remarkable influence on the performance of hydrolytic enzymes caused by the physical barrier restricting the enzymatic access to the sugar components and lignin-enzyme non-specific binding interactions (Rahikainen *et al.* 2013, Bornscheuer *et al.* 2014). For example, laccase and LMS have been

investigated as energy-saving pre-treatment step for detoxification of steam-exploded wheat straw to increase the digestibility of carbohydrates for bioethanol production (Moreno *et al.* 2016).

It has been found that non-phenolic β-O-4 lignin structures with α-carbonyl functionality are degradable with conventional pulping chemicals even at low temperatures (see e.g. Fearon *et al.* (2016). In pulp bleaching, alkali and hydroperoxide treatment are able to attack and degrade the non-phenolic lignin unit by degrading the propanoid side chain (Gierer *et al.* 1977, Gellerstedt and Agnemo 1980). Also TEMPO-based reagents have been found to effectively oxidize secondary and primary hydroxyls of lignin β-O-4 model compounds, giving corresponding ketones or aldehydes (Rahimi *et al.* 2013). Rahimi *et al*. (2015) further studied the reactivity of oxidized model compounds in reductive cleavage conditions, in the presence of, for example, zinc, aluminium, magnesium, iron or manganese, in aqueous formic acid at 110 ^{o}C. In the absence of reducing metal, the Cα-ketone was subjected to the formic acid/sodium formate reaction conditions at 110 ^{o}C and remarkable selectivity on cleavage of the β-ether bond with formation of corresponding diketones and phenols were obtained (Rahimi *et al.* 2015).

In white-rot fungi, a newly found class of enzymes are intracellular glutathione–S-transferases (β-etherases or GST-etherases), formerly reported for the bacterium *Sphingobium* ssp. SYK-6 (Gall *et al.* 2014), that cleave the β-O-4 linkage of dimeric α-keto-β-O-4 ether-linked substrates by nucleophilic attack on the β-carbon of the substrate using glutathione as co-substrate. The thioether adduct can be further released by specific glutathione lyases. These new class of enzymes are considered as potential candidates for lignin degrading catalyst in future biotechnical applications (Picart *et al.* 2015).

3. AIMS OF THE RESEARCH

The catalytic efficiency of the so-called 'natural' mediators needed to be evaluated in the Biorenew EU-project (White Biotechnology for added value products from renewable plant polymers: Design of tailor-made biocatalysts and new industrial bioprocesses). The project's aim was the use of biotechnology tools in order to obtain a new generation of tailor-made enzymes for the sustainable production of added value products using renewable plant polymers (cellulose and lignin from wood and other fibers) as feedstocks. The effect of phenolic mediators had been shown in the decolorization of recalcitrant dyes by laccase (Camarero *et al.* 2005) but knowledge of how lignin is modified using these co-catalysts was not available. The first task was to synthesize representative non-phenolic lignin model compounds (Figure 17, compounds **1**-**9**) for studies on LMS. Different phenolic compounds of natural origin, either commercial or derivatized by synthetic means (Figure 18, compounds **10**-**22**), were studied as mediators and the most promising ones were further selected as mediators in the studies with oxidoreductases.
The efficiency of different low redox potential (from ascomycete *M. thermophilia*) and high redox potential (from basidiomycete *Pycnoporus coccineus*, *P. sanguineus* and *P. cinnabarinus* ssp.) laccases with selected natural and conventional chemical mediators with representative lignin model compounds was evaluated to screen the applicability of these natural phenols on lignin modification. During the study the MnPs, known to effectively catalyze the oxidation of phenolic compounds, were also selected as catalysts to further examine whether the selected phenols equally worked as peroxidase mediators in the similar manner as with laccase. Two commercial fungal peroxidases, MnP from *Phlebia sp. b19* and VP from *B. adusta* were selected as catalysts. Finally, in order to test the oxidation system for polymeric substrates, a new oxidation procedure was developed to oxidize and fractionate synthetic lignin (syringyl-guaiacyl dehydrogenation polymer, SG DHP).

The main hypotheses of the work were:
1. Small molecular-weight mediator molecules are an integral part of the biochemical degradation of lignin, performed by lignocellulose-decomposing fungi.
2. Redox mediators may well be naturally occurring plant compounds, which are formed from lignocellulose through the action of lignin-degrading fungi, or the compounds are synthesized by the fungus.
3. The most efficient natural mediators described are the syringyl-type compounds, which originate e.g. from hardwood lignin.
4. It is possible to synthesize derivatives of natural mediators, which are more efficient than those of natural origin, such as acetosyringone, methyl syringate and other related compounds.
5. Non-phenolic lignin model compounds can be attacked and modified by LMS
6. Manganese peroxidase oxidizes phenolic mediators, producing phenolic radicals that can further oxidize non-phenolic lignin structures in a similar manner to laccases.
7. Phenolic mediators can be used to accelerate the oxidation of non-phenolic moieties of lignin by both manganese peroxidases and versatile peroxidases.
8. The production of hydrogen peroxide and organic peroxides 'in situ' by catalytic combustion of organic diacids can be used as an electron acceptor in the catalytic cycle of peroxidases.

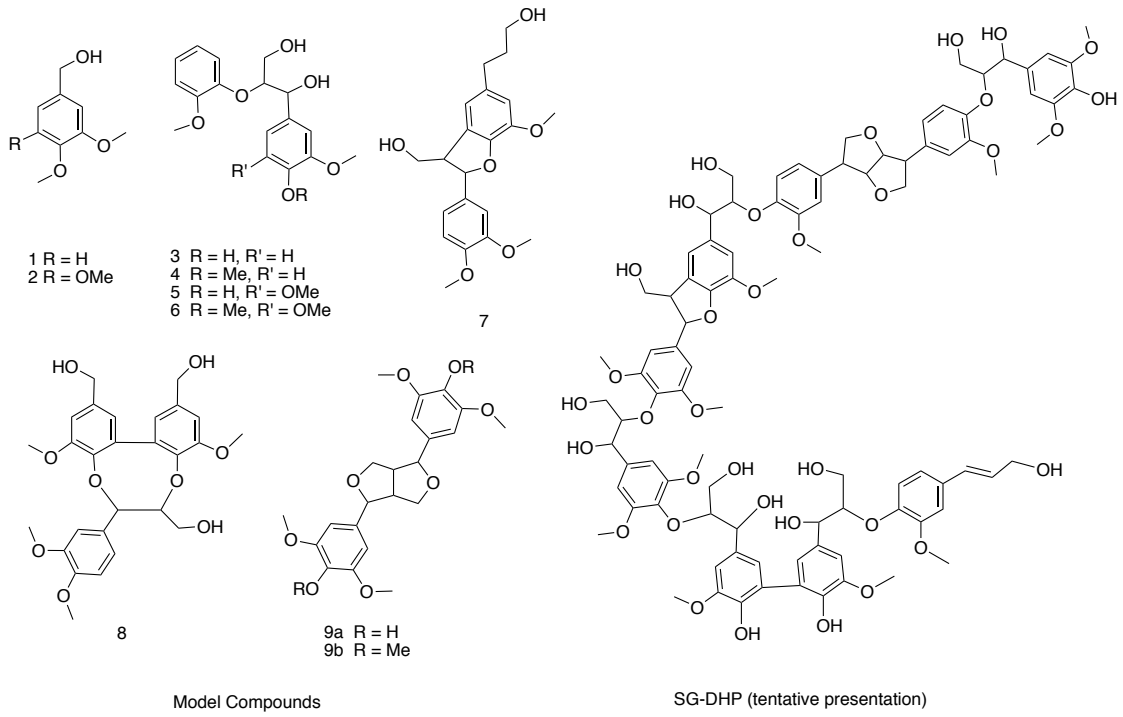


Figure 17. The lignin model compounds applied in the study. **1**, veratryl alcohol; **2**, 3,4,5-trimethoxybenzyl alcohol; **3**–**4**, phenolic (**3**) and methylated (**4**) guaiacylglycerol-β-guaiacyl ether dimers; **5**–**6**, phenolic (**5**) and methylated (**6**) syringylglycerol- β-guaiacyl ether dimers; **7**, methylated phenylcoumaran-type β-5 dimer (Me-PC); **8**, methylated dibenzodioxocin (Me-DBD); **9a**, syringaresinol (SR); and **9b**, methylated syringaresinol (Me-SR). SG-DHP is a tentative presentation of synthetic lignin, the guaiacyl-syringyl dehydrogenation polymer.

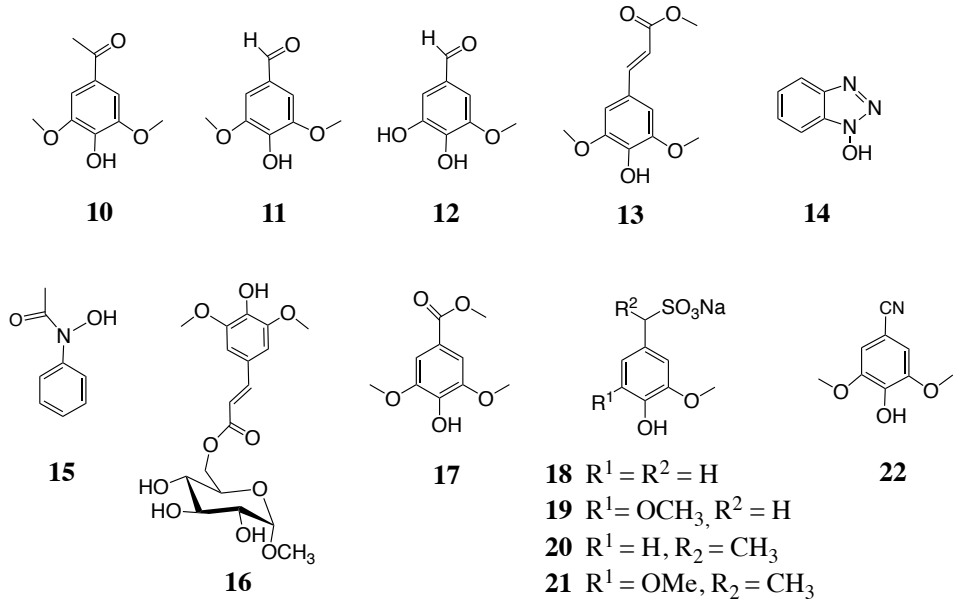
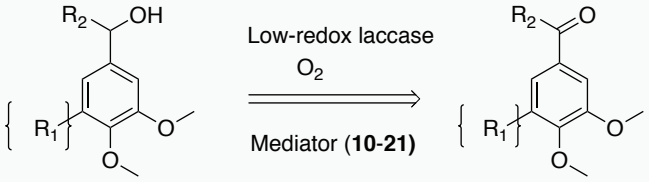


Figure 18. The mediators applied in the study. **10**, acetosyringone (AS); **11**, syringaldehyde (SA); **12**, 5-hydroxyvanillin; **13**, methyl sinapate; **14**, 1-hydroxybenzotriazole (HBT); **15**, N-hydroxyacetanilide (NHA); **16**, methyl 6-O-sinapoyl-α–D-glucopyranoside (Sglp); **17**, methyl syringate (MeS); **18**–**21**,

α-sulfonylated derivatives of vanillyl alcohol (**18**), syringyl alcohol (**19**), apocynol (**20**), 5-methoxy-apocynol **(21**) and **22**, 3,5-dimethoxy-4-hydroxybenzonitrile (SCN).

4. SUMMARY OF THE PUBLICATIONS

4.1 PUBLICATION I: LMS OXIDATION BY LOW REDOX *M. TERMOPHILIA*



Synopsis: *Simple syringyl phenols oxidize high redox potential lignin model compounds by low-redox potential laccase.*

In paper one the utility of simple syringyl-type phenols and some common synthetic compounds (**10-21**) were compared with regard to their ability to act as laccase mediators in the oxidation of non-phenolic lignin model compounds (**1-9**). The mediators were first screened using the assay of Reactive Black 5 decolorization. The results showed that methyl syringate (MeS) gave the highest decolorizing rate followed by acetosyringone (AS), and syringaldehyde (SA). Also, methyl sinapate and methyl 6-*O*-sinapoyl-α-D-glucopyranoside showed some mediating capacity, but vanillin and 5-hydroxy analogs proved to be not effective as mediators. The catalytic potential of commercial low-redox potential ascomycete laccase from hyperproducing *Myceliophthora thermophila* (provided by Novozymes) was examined with these phenolic mediators and some common synthetic mediators in order to modify lignin substructures. This was the first study on syringyl-type natural mediators using lignin model compounds with product analysis.

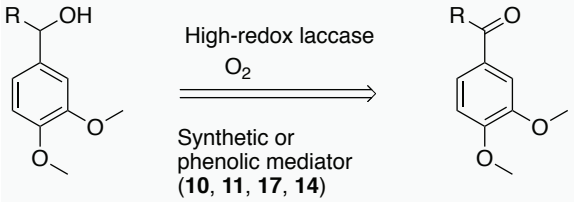
It was demonstrated that syringyl-type phenols, especially AS and MeS, were able to mediate the oxidation of substrates of high oxidation potential by a laccase with a low redox potential. A mediator dose of around 10% showed good performance compared to the equimolar quantities needed when the synthetic compounds HBT or NHA were used. On the other hand, at equimolar doses NHA oxidized the model compounds faster and more effectively than any of the mediators studied. The results suggest that such natural mediators are rather close in mediating property to those of commonly used synthetic mediators with low-redox laccases. The non-phenolic dimeric lignin model compound, the guaiacylglycerol β–guaiacyl ether adlerol (**4**), was oxidized much more slowly and with modest yield (5%). The syringaresinol compound (**10**) and the phenylcoumaran compound (**7**) were not oxidized but dibenzodioxocin compound (**8**) showed surprisingly good reactivity (30%) in the AS-laccase system.

The results also clearly demonstrated that SA and AS, but also NHA, reacted further during the oxidative reactions. The observed stabilities for the natural mediators in the reaction conditions applied with *M. thermophila* laccase were: MeS > AS >> SA. As a result, MeS proved as the most effective and most stable of the natural mediators in this study.

This result opened an interesting field for further evaluation over whether these syringyl-type phenolics could be of commercial value in the degradation of lignocellulosic materials.

Since then, the lignin degradation capacity of the *M. thermophila* laccase − MeS system in low mediator doses has been demonstrated e.g. in experiments with biobleaching eucalyptus pulp (Babot *et al.* 2011, Rico *et al.* 2014) and as a pretreatment step before saccarification of eucalyptus feedstock (Rico *et al.* 2014, Rico, Rencoret, del Río, *et al.* 2014).

4.2. PUBLICATION II: LMS OXIDATION BY HIGH-REDOX *PYCNOPORUS* SPECIES



Synopsis: *Oxidation efficiency of mediators can be enhanced by high-redox potential laccases. The difference in redox potentials affects the oxidation capacity.*

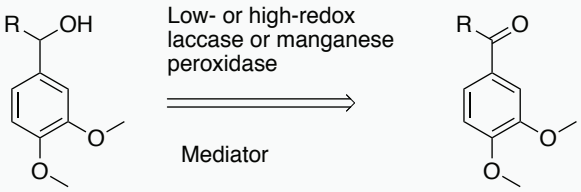
In paper II the aim was to screen the natural biodiversity in species *Pycnoporus coccineus* and *Pycnoporus sanguineus* for a new generation of laccases with properties suitable for the lignin-processing sector.

The wild-type laccases from species *Pycnoporus coccineus* and *Pycnoporus sanguineus* originating from subtropical and tropical environments (strains BRFM 938, BRFM 66 and BRFM 902 from INRA, Marseille) were studied. The three laccases (59.5–62.9 kDa with 7–10% carbohydrate content) had high redox potentials (0.72–0.75 V vs normal hydrogen electrode at pH 6) and they remained highly stable at up to 75–78°C and at pH 5–7 mixtures. The organic solvent tolerance of the selected laccases was tested and the results showed that they were resistant to methanol and ethanol, acetonitrile, 1,4-dioxane and dimethyl sulfoxide even at concentrations as high as 50% (v⁄v).

The biotechnological potential of the three selected laccases was assessed through the degradation of various polyphenolic dyes and the oxidation of non-phenolic lignin model compounds. In these studies, the mediative properties of AS, MeS and HBT were evaluated in the oxidation of non-phenolic lignin model compounds, the monomeric veratryl alcohol (**1**) and dimeric guaiacylglycerol β–guaiacyl ether, adlerol (**4**). It was found that HBT, AS and MeS efficiently mediated the oxidation of veratryl alcohol (32–100%) by these laccases. The synthetic mediator HBT was the most effective in the oxidation of both veratryl alcohol (100%) and adlerol (86%) by BRFM 66 laccase, which exhibited the highest redox potential. AS was found to be as effective as MeS, with the oxidation of veratryl alcohol 30–40% after 48 h of incubation with all three laccase systems. Adlerol was significantly oxidized only by laccase-HBT systems, yielding 33–86% of adlerone, that is 10-17 times more than by using laccase-'natural' mediator systems. Marked differences were thus found in the oxidation of veratryl alcohol and adlerol by the three enzymes. The reactivities were in the following order: BRFM 66 > BRFM 938 > BRFM 902 for veratryl alcohol, and BRFM 66 > BRFM 902 > BRFM 938 for adlerol. This study identified *P. coccineus* and *P. sanguineus* as outstanding producers of high-redox potential laccases, i.e. easy to purify and scale-up for industrial production. Three new laccases proved to be suitable models for white

biotechnology processes and for further molecular breeding in order to create a new generation of tailor-made enzymes.

4.3. PUBLICATION III: COMPARATIVE STUDY OF DIFFERENT OXIDOREDUCTASES AND THEIR MEDIATED OXIDATION CONDITIONS



Synopsis: *The effect of pH for syringyl-type phenol mediated reactions is crucial. The phenolic mediator effect may also work in manganese peroxidase catalyzed reactions.*

In paper III the oxidation capacity of laccases was further investigated using laccase from *M. thermophila* and *Pycnoporus cinnabarinus* ss3 laccase from the monokaryotic hyperproducing strain ss3 with comparison to the wild-type laccases from *P. coccineus* BRFM 938, *P. sanguineus* BRFM 902 and *P. sanguineus* BRFM 66 from INRA, Marseille. Also a preliminary study for utilization of manganese peroxidase from *Phlebia* sp. *Nf* b19 as catalyst using syringyl-type phenols as their mediators was assessed with comparison to the well-known lipid peroxidation mediator system.

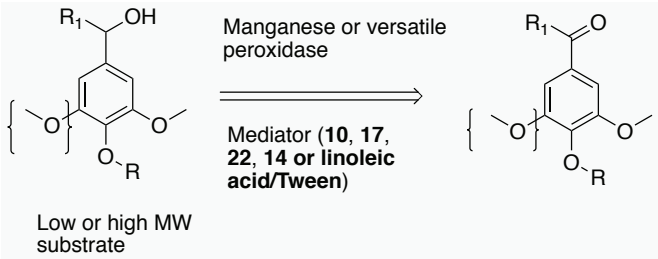
The results demonstrated that the action and oxidation efficiency of both low-redox and high-redox potential laccases are enhanced by using plant-derived syringyl-type phenols as mediators; AS and MeS in particular effectively mediated the oxidation of comparatively high oxidation potential substrates even with a low-redox potential (0.48 V) *M. thermophila* laccase. Even though HBT has been reported one of the most effective laccase mediators, the low activity with *M. thermophila* is most likely because the redox potential of the enzyme is too low to effectively oxidize this redox mediator (Li *et al.* 1999). Syringyl-type phenolic compounds on the other hand possess lower redox potential due to their electron-donating substituents thereby making their enzymatic oxidation easier by a low-redox laccase.

The results showed that the efficiency of these mediators can still be enhanced by using high redox potential (0.75 V) laccase e.g. *P. cinnabarinus ss3*. The oxidation of veratryl alcohol (**1**) to veratraldehyde by the high redox potential laccase *P. cinnabarinus* ss3 was effectively mediated by HBT, NHA, AS and MeS resulting in 40-100% conversion. With this high-redox potential catalyst, HBT was clearly the most effective of the studied mediators, especially with the dimeric lignin model compound, adlerol (**4**). In these reaction conditions adlerol was found to oxidize to the corresponding α−carbonyl product, adlerone, with 90% conversion without any Cα−Cβ cleavage of the propyl side chain. This result showed, that HBT indeed oxidizes the lignin substrates preferably using HAT mechanism. The high performance of synthetic mediator HBT clearly demonstrated that further improvement on the stability of the 'natural' mediators is required. Also, it was clear that the side products formed by oxidation of phenolic mediators should be studied further.

Finally, the effect of pH (at range 4-6) was evaluated on the performance of the phenolic mediators on veratryl alcohol oxidation with laccase from *P. cinnabarinus* ss3 as a catalyst (that has an optimum pH of 4). The study showed that the effect of pH for the reaction is crucial when exploiting phenolic mediators and e.g. the critical pH for oxidation reaction catalyzed by laccase from *Pycnoporus cinnabarinus* ss3 is around 5.5. Lower pH in the reaction medium leads the mediative oxidation reactions towards degradation of the mediator by acid-catalyzed side reactions where the mediator is consumed to give the corresponding *p*-benzoquinone and most likely dimers and polymerized polymeric products as well. The results suggested that the optimum pH for the oxidation of the target substrate was influenced by the stability of the oxidized mediator, rather than the optimal pH for laccase.

In order to study the effect of different mediators in the MnP-induced oxidation reactions, by *Phlebia* sp. Nf b19 (*Nematoloma frowardii* b19), three oxidation systems were studied: lipid peroxidation, Mn(III) malonate chelate with in situ peroxide production by glucose oxidase and a system with phenolic mediators where hydrogen peroxide was generated directly from oxalic acid in malonate buffer. The results suggested that also MnP-catalyzed reactions by *Phlebia* sp. *Nf* b19 were likewise facilitated by the presence of syringyl-type mediators and the system was much more efficient than the MnP-lipid peroxidation system.

4.4. PUBLICATION IV: MEDIATED OXIDATION BY MNP FROM *PHLEBIA SP. B19* AND VP FROM *B. ADUSTA*



Synopsis: *The fungal peroxidases readily catalyze the oxidation of syringyl-type phenols, that transfer the oxidation further to non-phenolic lignin model compounds or synthetic lignin.*

In this study we investigated further our earlier finding that fungal peroxidases readily catalyze the oxidation of syringyl-type phenols in a similar manner to laccases, with the formation of reactive radical species to transfer the oxidation further to the non-phenolic compounds. For this purpose two fungal peroxidases, MnP from *Phlebia sp. b19* and VP from *B. adusta* (both commercial peroxidases from Jenabios, Germany) were selected as catalysts and the behavior of the MnP/Mn(II) oxidation system, as well as lipid peroxidation systems were followed with and without the presence of simple organic mediators (MeS, AS, SCN (3,5-dimethoxy-4-hydroxybenzonitrile) and HBT). The final oxidation targets were non-phenolic monomeric veratryl alcohol (**1**) and dimeric synthetic lignin model compound adlerol (**4**) and syringyl-guaiacyl-type synthetic lignin (SG DHP).

In all reaction systems the initiation of the oxidation occurred autocatalytically to generate peroxides through the action of the reactive Mn(II)/Mn(III) pair. The reaction system was found to oxidize the activated carboxylic acids, e.g. oxalic and linoleic acid that subsequently produced activated oxygen species to fuel the catalytic cycle of peroxidases and to participate in various oxidation processes, without leading to the inhibition of the peroxidases.

It was found that the phenolic mediators AS, MeS and SCN clearly enhanced the MnP catalyzed oxidations. Both reaction systems, generating either aryloxy radicals or alkylperoxy radicals by the chelated Mn(III) oxidized monomeric veratryl alcohol (**1**) and dimeric β–O-4 type adlerol (**4**) produced selectively the corresponding α–carbonyl compounds. No Cα-Cβ-cleavage of the dimeric adlerol was detected in MnP catalyzed reactions whereas in some instances the VP catalyzed oxidations gave some veratryl alcohol indicating LiP-type activity. It was also found that the yields in the oxidation of veratryl alcohol were considerably higher in the reactions mediated with MeS, SCN and AS than in the lipid peroxidation system. In contrast, the lipid peroxidation system gave higher yields in the oxidation of dimeric adlerol. Also, the VP from *B. adusta* gave better yields (19-29% within 72 h) than the MnP from *Phlebia sp. b19* (6-16% within 72 h) with phenolic mediators. With VP from *B. adusta* as catalyst, MeS was found more effective than AS, with up to 42% conversion of veratryl alcohol to veratraldehyde within 120 h compared to 36% with AS.

Finally, we developed a new type of reaction system for oxidation studies of synthetic lignin (DHP). The DHP was synthesized as slurry with cellulose, therefore more resembling natural lignocellulose, and this cellulose bound DHP material was treated as substrate in an enzymatic-mediator –system. The reaction system allows simple isolation of low and high molecular weight fractions for the analysis by simple solvent extraction procedure. The resulting polymers were analyzed using size exclusion chromatography (SEC) and nuclear magnetic resonance spectroscopic (^{1}H, ^{13}C, 2D NMR) methods, and finally the changes in different types of hydroxyl groups indicated by ^{31}P NMR analyses were compared. The results suggest, that the treatment of SG-DHP in different oxidative conditions by peroxidases can markedly reduce the average molecular weight of the samples. The ^{31}P-NMR analysis showed that the oxidation occurred particularly in the phenolic syringyl end-units, leading to the accumulation of guaiacyl structures in the materials.

The amount of condensed groups was found to decrease in the oxidation reactions, suggesting that some of the reactions occurred also in the condensed β-5 sites of the polymers and that no repolymerization to biphenylic structures took place. In the structural analysis of oxidized DHPs by ^{1}H and ^{13}C-NMR spectroscopy, structural deformations in the propyl side chain area were found as suggested by the reduced amounts of β-O-4 ether and phenylcoumaran (β-5) structures.

4.5 CONCLUDING REMARKS

The research on lignin modifying and degrading enzymes, basidiomycetous oxidoreductases has recently revealed that these enzymes are able to use small lignin derived compounds as redox shuttles to get access to the lignin imbedded into the plant cell wall. In this thesis work was evaluated the ability of fungal laccases and peroxidases to catalyze indirectly the oxidation of the persistent lignin substructures, especially those mimicking arylglycerol–β-aryl ether structures, by using lignin-derived syringyl-type phenols as co-oxidants. The efficiency of different low-redox potential (from ascomycete *M. thermophilia*) and high-redox potential (from basidiomycete *Pycnoporus coccineus*, *P. sanguineus* and *P. cinnabarinus* ssp.) laccases with selected natural and conventional chemical mediators with representative lignin model compounds was evaluated in order to screen the applicability of these natural phenols on lignin modification. It was found that especially methyl syringate and acetosyringone mediated the laccase-catalyzed oxidation even with low-redox potential laccase that generally shows only fair oxidation activity on lignocellulosic oxidation. Also, the mediating rate and effectivity were enhanced by using high redox potential laccases and suitable reaction conditions to retain the stability of the mediator. The oxidation efficiency and chemical stability of the mediator was shown to depend on the pH of the reaction medium that should be optimized for each catalyst. These simple phenolics were, for the first time, also proved to mediate the MnP and VP catalyzed reactions of non-phenolic compounds. Significantly, no Cα-Cβ-cleavage of the dimeric adlerol was detected in laccase or MnP catalyzed reactions whereas in some instances the VP catalyzed oxidations gave some veratryl alcohol indicating LiP-type activity. In all cases, mediated reactions gave selective α–oxidation products of benzyl alcohol moieties. To prove the effect also on polymer material, oxidation experiments were also performed with synthetic lignin and the structural analysis of the oxidized polymers showed clear modifications in the polymer outcome e.g. structural deformations in the propyl side chain area and, the overall molecular weights of the polymers were decreased. These oxidations provide valuable intermediates for further treatments in various lignin valorization processes. In the near future, this oxidative laccase-mediator oxidative system will be explored in an EU Horizon 2020 project for valorization of biorefinery lignin by combination of biotechnical and chemical means to added-value products such as marine biofuel, engine octane boosters and valuable low-molecular weight chemicals.

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